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CONTENTS

Antifungal properties and phytochemical screening of crude extract of *Lemna pauciscostata* (Helgelm) against fish feed spoilage fungi.

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Abstract

Aqueous and ethanolic extracts of duckweed (*Lemna pauciscostata*) meal was tested on fungal isolates from stored pelleted fish feeds to ascertain its efficacy as an antifungal agent against feed spoilage fungi. Test organisms used were *Fusarium oxysporium, Penicillium digitatum, A. niger, A.flavus, A.fumigatus, Rhizopus oryzae and R.stolonifer.* Phytochemical analysis of the crude extract was also conducted to determine the active ingredients in duckweed meal. Proximate nutrient composition and amino acid analysis to determine the suitability or otherwise of duckweed meal as a feed additive was also carried out. Results showed that ethanolic extracts exhibited higher antifungal properties with total growth inhibition in some test organisms than the aqueous extract. However the efficacy of the extracts against fungal growth increased with increase in concentration. Result of the phytochemical analysis of duckweed meal revealed the presence of tannins and steroids. Determination of the proximate nutrient composition and amino acid analysis also showed that duckweed meal is rich in essential nutrients. [Life Science Journal 2010;7(3):1-4]. (ISSN: 1097-8135).

Key Words: Duckweed meal, antifungal, extract, ethanolic, aqueous.

Introduction:

Antimicrobial agents, including food preservatives have been used to inhibit food borne fungi and extend shelf life of processed food for many centuries. Many naturally occurring compounds found in edible and medicinal plants, herbs and spices have been shown to posses' antimicrobial functions and could serve as agents against food spoilage micro-organisms (Deans and Ritche, 1987; Janseen et al., 1985). Tannins and steroids have been shown to possess antimicrobial ability against several food spoilage fungi (Lucia et al., 2002; Costa et al., 2000).

Lemna, a group of tiny, free-floating vascular plants with worldwide distribution are found in small water bodies such as fishponds, ditches and lagoons, which are nutrient rich. Their ability to bloom within days after cultivation with high nutrient content has made them a rich source of food nutrients in the diet of fishes and animals alike. Lemna have been shown to exhibit antimicrobial activity (Skilicorn et al., 1993; Mbagwu, 2001).

A major problem in fish feed production is associated with storage. A lot of losses occur in feedstuff during storage. Fungal attacks along with other kinds of storage problems are responsible for unreasonable losses occurring in feedstuff during storage. Such losses are loss in weight, loss in quality of feed and health risks to fish that feed on infected feed.

The addition of some fungicides could suppress the growth of fungi in feed, however as with all pesticides, these chemicals are likely to have side effects which may be hazardous to fish health. Therefore if a non-hazardous process could be found capable of suppressing or even eliminating fungal growth in stored compound feed, it would be of immense practical and economic benefits to the aquaculture industry in Nigeria.

Fungal isolates from stored pelleted fish feeds were therefore utilized as test organisms on extracts of *Lemna pauciscostata* used directly as fish feed.

Materials and Method

Samples of duckweed (*Lemna pauciscostata*) were harvested from the outdoor concrete tanks of National Institute of Freshwater Fisheries Research Hatchery Complex, New Bussa, Nigeria. They were thoroughly rinsed with clean water and evenly spread on a mosquito net-size mesh to dry and thereafter dried in a forced air oven at 65^0C for 48 hours before being grounded to powder with a milling machine (Mbagwu and Adeniyi, 1988). The powder was exhaustively extracted with 95% Ethanol and sterile distilled water at room temperature for 2 days.

Extracts were filtered and the solvent removed under reduced pressure at 40^0C (Souza et al; 2002). Preliminary antifungal assays were performed using seven test organisms and extracts at concentrations of 5% and 10% respectively. Control plates had 95% ethanol and sterile distilled water without extracts. Mycelial plugs of the test organisms measuring 5.0mm in diameter were cut with sterile cork borer from the advancing margin of the fungal colonies and placed at the centre of Potato Dextrose Agar (Adedayo, 1994).

All plates were incubated at 25^0C and radial mycelial growth recorded.

Dried duckweed was ground using Automatic weed Grinder (Scientific Instrument, Yoshida Seikusho Co. Ltd, Tokyo, Japan, No. 5678).

Extracts for phytochemical analysis were concentrated to dryness in hot air oven at 45^0C (Odebiyi and Sofowara, 1978). The dried extracts were tested for alkaloids, saponins, tannins, anthraquinones, flavonoids,

steroids and phlobatannins (Harbone, 1984). Proximate composition of the following nutrients was determined using standard procedures of AOAC (2000): moisture, crude protein, lipid, crude fiber and Nitrogen free extract (NFE). Amino acid profile of duckweed meal was determined using the method of Abdullahi (2001).

Results and Discussion

Differential efficacy on the test organisms was noted between the aqueous and ethanolic extracts of *Lemna pauciscostata* (Table 1). Ethanol appeared a better extractant judging from the wider activity spectrum and the effect of its extract on isolates. This observation perhaps suggests the possibility of the occurrence of bioactive substances that are not only soluble in water but also in organic solvent in the plant material.

Majekodunmi et al., (1996) and Martinez et al., (1996) reported that a higher activity of extractable natural products was obtained in ethanol compared with aqueous extracts. Ahmed et al., (1998) also observed that alcoholic extracts showed greater activity than the aqueous and hexane extract of some Indian medicinal plants with antimicrobial properties. While ethanolic extracts showed total growth inhibition on some organisms even at 5% concentration, aqueous extract showed none although; growth rate was slower at the 10% aqueous than 5% aqueous extract. The most susceptible isolates to both the aqueous and ethanolic extracts were *Aspergillus fumigatus* and *Fusarium oxysporium* where total growth inhibition was observed.

Several authors have reported on the antimicrobial activity of various plant extracts using different means of extraction on various plants materials. Natarajan et al., (2005) reported the antifungal properties of three medicinal plant extracts against *Cercospora arachidicola*. They reported that fungal growth was gradually suppressed with increasing extract concentration. Similar findings have been reported by Lucia et al., (2002) on the antifungal properties of Brazilian cerrado plants. They stated that ethanolic extracts of the plants showed higher antifungal activity.

Silva et al., (2001) and Costa et al., (2000) also reported the antifungal activity of extracts of *Eugenia dysentrica* and *Annora crassiflora* against some pathogenic fungi. The findings from this study are similar to the report of these authors.

Adekunle and Ikunmapayi (2006) working on the antifungal properties and phytochemical screening of the crude extracts of *Funtumia elastica* and *Mallotus oppositifolius* reported varying degrees of antifungal activity of the plant extracts on some test organisms including *Aspergillus flavus* and *Penicillium* species. The same authors reported that the ethanolic extracts of the plants showed higher antifungal activity on the test organisms than the aqueous extracts.

The result of the phytochemical screening of the *Lemna pauciscostata* extracts (Table 2) revealed the presence of tannins and steroids. Research findings from several authors have shown that both tannins and steroids possess antimicrobial ability. Bairagi et al., (2002) reported the presence of tannins and phytic acid in duckweed meal. Baba Moussa et al., (1999) reported antifungal activities of seven West African combretaceae extracts used in traditional medicine against several fungal species. The result of the phytochemical screening of these plant extracts showed that they were rich in tannins and saponins.

Adekunle and Ikunimapayi (2006) reported the presence of tannins,, saponins and steroids among other substances from the extracts of *Funtumia_elastica* and *Mallotus oppositifolius* which they inferred were likely to be responsible for the antifungal activity exhibited by these plants.

Other authors have also reported similar findings (Onadapo and Owonubi, 1993; Barnabars and Nagarajan, 1988; Adekunle et al., 2003; Subhisha and Subramoriam, 2005; Adio et al., 2004).

Barapedjo and Bunchoo (1995) implicated these phytochemicals to inhibit cell wall formation in fungi leading to the death of the organisms.

The findings of this experiment are similar to the report of these authors.

The results of the proximate composition of nutrients as well as that of amino acid profile in Tables 3 and 4 respectively showed that duckweed meal is rich in essential nutrients. Therefore, incorporating it into fish feed formulation will not cause any negative effect to fish growth and survival. Several authors have reported the use of duckweed as fish feed ingredient (Fasakin and Balogun, 1998; Fasakin et al., 2001; Fasakin et al., 1999; Edwards, 1980; Robinette et al., 1980).

Conclusion

From the findings of this experiment, there are indications that duckweed meal could be incorporated into formulated fish feeds to serve as antifungal agent against feed spoilage fungi. This will be of immense benefit to the local fish farmers and therefore improvement in the fisheries aquaculture practice in Nigeria.

Table 1: Efficacy of duckweed extracts on the mycelial growth of fungal isolates after 72 Hours.

TEST ORGANISMS	MYCELIAL GROWTH IN MM					
	AQUEOUS EXTRACT EXTRACTS			ETHANOLIC		
	0%	5%	10%	0 %	5%	10%
Fusarium oxysporium	46	21	10	10	-	-
Penicillium digitatum	50	35	24	9	5	-
Aspergillus niger	47	27	18	16	7	2
Aspergillus fumigatus	38	18	12	4	-	-
Aspergillus flavus	50	38	20	16	-	-
Rhizopus oryzae	36	29	16	14	-	-
Rhizopus stolonifer	42	21	13	22	10	4

Table 2:Phytochemical analysis of duckweed meal.

Test	Result
Alkaloids	_
Saponins	_
Tannins	+
Anthraquinones	_
Flavonoids	_
Phlobatannins	_
Steroids	+

Table 3: Proximate composition of duckweed (*Lemna pauciscostata*) meal

Sample	% Crude Protein	% Ether Extract	% Ash Content	% Moisture Content	% Crude Fiber
Duckweed	34.18	5.3	13.55	2.8	14.28

Table 4:Amino acid analysis of duckweed meal.

AMINO ACID	AMOUNT
LYSINE	5.30
HISTIDINE	2.03
ARGININE	4.25
ASPARTIC ACID	6.87
THREONINE	4.81
SERINE	3.89
GLUTAMIC ACID	10.11
PROLINE	3.08
GLYCINE	4.87
ALANINE	1.91
CYSTEINE	1.21
VALINE	4.24
METHIONINE	1.01
ISOLEUCINE	4.81
LEUCINE	6.30
TYROSINE	3.04
PHENYLALANINE	4.26

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Chronic Exposure Of Dicofol Promotes Reproductive Toxicity In Male Rats

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ABSTRACT

Dicofol is an organochlorine acaricide widely used in local market. The present study was conducted to evaluate how far dicofol chronic toxicity affects male fertility indices, as well as for assessment of reproductive toxicity which may result from this acaricide by estimating the sexual and reproductive hormones. Moreover, to investigate the effect on testicular function and epididymal oxidative parameters. In this investigation, two equal groups of male albino rats were orally administered dicofol, at 4.19 and 16.75 mg/kg body weight/day through drinking water (30 and 120 part per million, respectively) for consecutive 90 weeks. Dosages represent $^1/80$ and $^1/20$ LD_{50} of dicofol, respectively. The third group was kept as control group. At the end of each experimental period (16, 28 and 90 weeks), blood samples were taken for estimation of sexual , reproductive and thyroid hormones. Also, animals were dissected and the reproductive organs (epididymus and testes) were taken to measure fertility indices, oxidative parameters and testicular biomarkers. The main results of this study were : dicofol at both doses (lower and higher) decreased testes and epididymus weights, this effects were dose-related and associated with decline in epididymus sperm count, percent of sperm motility, viability and maturity and increased abnormal sperm morphology. Moreover, decline in serum testosterone, follicle stimulating hormone and luteinizing hormone levels concomitant with an elevation in estradiol and progesterone levels were observed. Additionally, Dicofol-treated rats demonstrated de-generation and atrophy of some seminiferous tubules associated with depression in luminal spermatozoal concentration. Meanwhile, dicofol increased oxidative stress by an elevation lipid peroxidation index associated with depletion in glutathione level. Concerning the testicular biomarkers, dicofol increased total protein level and decreased the activities of the enzymes responsible of spermatogenesis, *i.e.* lactate dehydrogenase, acid and alkaline phosphatase activities. Conclusion: the results reinforce the idea that, dicofol, as o'ch pesticide, possesses estrogenic and antiandrogenic properties as well as oxidative stress. [Life Science Journal 2010;7(3):5-19]. (ISSN: 1097-8135).

Keywords: Rats, pesticide, dicofol, chronic toxicity, fertility, testes, epididymus, lipid peroxidation, glutathione, testicular markers, hormones.

1. INTRODUCTION

Pesticides are agricultural chemicals used for controlling pests on the plant or animals. Problems associated with pesticide hazards to man and environment are not confined to the developing countries, but extended to developed nations and still facing some problems in certain locations **(Nuckols *et al.*, 2007)**.

The severity of pesticide hazards is much pronounced in third world countries. A number of long persistent organochlorines (O`Ch), which have been banned or severely restricted are still marketed and used in many developing countries **(Hajjo *et al.*, 2007).**

The ideal pesticide is a pesticide which be effective only against the pests and be harmless to people, animals and environment. However, they have some side / non-target effects that may show undesired actions appears latter **(El-Kashoury *et al.*, 2005)**.

A large number of chemicals occurring in our environment may have potential to interfere with the endocrine system of animals **(Dalsenter *et al.*, 1997)**. Many of these chemicals can disrupt development of the endocrine system and of the organs that respond to endocrine signals in organisms indirectly exposed during prenatal and/or early postnatal life; effects of exposure during development are permanent and irreversible **(Colborn *et al.*, 1993).** Several pesticides have been reported to produce gonadal toxicity, among these are persistent and bioaccumulative organochlorine pesticides (O`Ch). Increasing interest has been observed among environmental and health institutions regarding the potential reproductive effects due to exposure to occupational and environmental chemicals **(Dalsenter *et al.*, 1997).**

Over the past decade, there has been an increasing focus on the effects of synthetic chemicals on human endocrine system-specially on effects related to androgen and estrogen homeostasis **(Boas *et al.*, 2006).**

An understanding of the developmental consequences of endocrine disruption in wildlife can lead to new indicators of exposure to endocrine disrupting contaminants. Thus, wildlife serve as important sentinels of ecosystem health, including human public health **(Guillette, 2000).**

There is much concern that exposure to such environmental contaminants causes decreased sperm

counts, impairment of sperm motility, reduced fertilization ability, producing abnormal sperm in men and wildlife (Alm et al., 1996).

It has been reported that, pesticides with such properties have been shown to cause overproduction of reactive oxygen species (ROS) in both intra- and extracellular spaces, resulting in a decline of sperm count and infertility in wildlife and human (Sharpe and Skakkebaek, 1993). The antioxidant system plays an effective role in protecting testes and other biological tissues below a critical threshold of ROS thus preventing testicular dysfunction (Oschsendorf, 1999). ROS has been shown to damage macromolecules, including membrane bound polyunsaturated fatty acid (PUFA), causing impairment of cellular function (Lenzi, 2000). Spermatozoa are rich in PUFA, and, therefore, could be highly susceptible to oxidative stress.

Dicofol, an organochlorine acaricide, is used widely on agriculture crops and ornamentals and in or around agricultural and domestic buildings for mite control (Ellenhorn *et al.,* 1997). It tends to accumulate in steroid producing organs such as adrenal gland, testes and ovary (Jadaramkunti and Kaliwal, 1999), and has antispermatogenic and antiandrogenic properties (Jadaramkunti and Kaliwal, 2002).

Previous studies have suggested that O`Ch, pesticides impaired the testicular functions through altering the activities of revelant enzymes (Sinha et al., 1995; Chitra et al.,1999).

Studies have been conducted on reproductive toxicity of dicofol in male rats following short-term exposure (Jadaramkunti and Kaliwal, 1999 and 2002). However, few and olden literature in this respect following long-term exposure specifically in mammals are available (larson, 1957 and Hazelton and Harris, 1989).

For the above mentioned, the objective of the current study was to characterize the endocrine-disrupting effects and reproductive toxicity of chronic daily exposure to dicofol in male albino rats via evaluation of male fertility indices and reproductive and sexual hormone levels. As well as oxidative parameters in cauda epididymus and testicular functions were studied.

2. Materials and Methods

2.1 Experimental Animals

In the present study, a total of seventy two male Wistar albino rats, *Rattus norvegicus* were obtained from the Farm of General Organization of Serum and Vaccine, Egypt. Male rats initially weighing 150±10 g were used. Animals were acclimated to holding facilities for two weeks prior to the experiment. The rats were housed in groups and kept in room under controlled temperature (24°C), humidity (30-70 %) and light (12: 12 hr / light: dark). All animals were provided balanced diet throughout the experimental period, these diet were obtained from Agricultural-Industrial Integration Company, Giza. Which formed of proteins, lipids, fibers, wheat, clover, maize, beans, crushed bones, molasses, choline, lysine, methionine, NaCl, Mn, Zn, Co, Mg, Cu, Fe, Se, I2 and many vitamins like A, E, D3, K, B1, B2, Biotin, B6, B12, Niacin and Folic acid. Animals were given food and water *ad libitum.*

2.2 Experimental Materials:

Dicofol (an O'ch pesticide), formulation 18.5 % Emulsifiable Concentrate (EC), was received from El-Nassr Company, was used through this investigation. Commercial name (Kelthane). The oral median lethal dose (LD50) of dicofol (administered to rats *per* OS.) was 348.86 mg/kg b.w, according to Weil's method (Weil, 1952). In this investigation, the used dosages were chosen according to the maximum tolerated dose (MTD), which suppressed body weight gain slightly i.e.; 10 %, generally ¼ MTD and 1/16 MTD, are then selected for testing (Hayes, 1989). Accordingly, dicofol was administered to rats at 4.19 and 16.75 mg/kg body weight (representing 1/80 and 1/20 LD_{50}) in drinking water.

2.3 Experimental Design

Seventy-two adult albino rats were allocated into three groups- 24 each - and treated with dicofol through drinking water for 90 successive weeks as the following:

Group (1): Rats received tap water only as an emulsifier of the pesticide dicofol (Emulsifiable concentrate).

Group (2): Rats received 4.19 mg/kg b.w. /day (30 ppm dicofol, which represents the lower dose).

Group (3): Rats received 16.75 mg/kg b.w. /day (120 ppm dicofol, which represents the higher dose).

dicofol emulsified daily in drinking water in glass bottle, and the bottles were cleaned daily.

All animals were observed at least once daily for behavior; signs of intoxications, mortality, morbidity, and food and water consumption were monitored daily. Animals weighed weekly and the dose was adjusted accordingly.

2.4 Sampling

2.4.1 Blood samples:

At the end of each experimental period, (16, 28 & 90 weeks), blood samples were collected, from fasted rats (control and treatanimals), from the orbital sinus vein using anesthetic ether by heparinized capillary tubes in plain tubes, according to Schalm, (1986) and allowed to be clotted at room temperature to obtain serum for hormonal assay

2.4.2 Sacrifice and tissues preservation:

Five animals/group were sacrificed by design 16, 28 and 90 weeks on study.

Testes and epididymus from sacrificed rats were removed immediately, clean of adhering tissues and weighed. Then, epididymus prepared for fertility evaluation and determination of oxidative biomarkers. Testes samples were taken for histopathological examination through the light microscope (Banchraft et al., 1996). And estimation of testicular functions.

2.5 Data Collection Techniques:

2.5.1 Evaluation of Fertility:

Spermatozoa were obtained by mincing the cauda epididymus in a known volume of physiological saline (w/v) at 37°C for evaluation of semen parameters under microscope (40X) as the following:

a) Sperm concentration (count)

The spermatozoa concentration was carried out by diluting the sperm suspension with water (1: 20), then mixed together, after that a drop of them delivered into the Neubaure haemocytometer in each side of the counting chamber. The haemocytometer is allowed to stand for 5 min. for sedimentation, then sperms were counted in the large five squares and expressed as sperm concentration in million, according to Feustan et al. (1989).

b) Sperm motility

The motility of sperm was evaluated directly after mincing in drop of sperm suspension, microscopically. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as percent of motile sperm of the total sperm counted, according to Linder et al. (1986).

c) Sperm viability by Eosin stain

This technique is used to differentiate between live and dead sperms. A drop of the Eosin stain added into sperm suspension on the slide and stand for 5 min. at 37°C, then examined under microscope. The head of dead spermatozoa stained with red color. While, the live spermatozoa unstained with Eosin stain. Sperm viability was expressed as percentage of live sperm of the total sperm counted, according to Krzanowska et al. (1995).

d) Sperm maturation by aniline-blue:

Nuclear maturation was evaluated by aniline-blue stain, according to Morel et al. (1998). Sperm nuclei that stained with blue color were considered to be immature. But nuclear mature sperm was not stained with aniline-blue. The percentage of immature sperm was calculated from the observation of one hundred sperm preparation from each group.

e) Sperm morphology

A drop of Eosin stain was added to the sperm suspension and kept for 5 min. at 37°C. After that a drop of sperm suspension was placed on a clean slide and spread gently to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology, according to the method of Feustan et al. (1989). The criteria chosen for head abnormality were; no hook, excessive hook, amorphous, pin and short head. For tail, the abnormalities recorded were; coiled flagellum, bent flagellum, bent flagellum tip. The result are the percentage overall abnormal form.

2.5.2- Hormonal assay:

a) Determination of serum testosterone:

Testosterone determination was performed according to the method adopted by **Jaffe and Behrman (1974)**, by using the coat-A-count technique,(radioimmunoassay)

b) Determination of serum estradiol:

Estradiol determination was performed according to the method of **Xing *et al.* (1983)**, by the coat-A-count technique (radioimmunoassay**).**

c) Determination of serum progesterone:

Progesterone determination was performed according to **Yalow and Berson (1971)**, by the coat-A-count technique (radioimmunoassay).

d) Determination of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH):

Follicle stimulating hormone and Luteinizing hormone determination Were performed according to **Santner *et* al. (1981)**, by the coat-A-count technique ,(immunoradiometric assay).

e) Determination of serum total thyroxine (T_4) and total tri-iodothyronine (T_3):

Thyroxine and tri-iodothyronine determination were performed in serum according to the method adopted by **Britton *et* al. (1975)**, by the coat-A-count technique, ,(radioimmunoassay)

2.5.3- Oxidative biomarkers in epididymus:

After evaluation of fertility related parameters, the remaining sperm suspension was collected and centrifuged by using cooling centrifuge. Then, the supernatant separated and kept at (-40°C) until determination of oxidative biomarkers.

a) Lipid peroxidation assay:

Lipid peroxidation (LPO)was measured in epididymus homogenates accordingly to the method of Ohkawa *et* al. (1979). Based on the formation of thiobarbituric acid reactive substances (TBARs) and expressed as the extent of malondialdehyde (MDA) production.

b) Determination of total glutathione (GSH & GSSG):

Principle:

Total glutathione (GSH) was measured according to the method of Bergmeyer and Graβl (1995) is based on the catalytic action of glutathione is a system, in which GSH undergoes periodical oxidation by DTNB and reduction by NADPH. The measure of the concentration of glutathione in samples is the velocity increase of absorbance (but not the end value).

2.5.4- Testicular functions:

A 10 % homogenate (W/V) of testes was prepared in ice cold normal saline using a chilled glass-teflon porter-Elvehjem tissue grinder tube, then centrifuged at 10,000 xg for 20 min. at 4°C. The supernatant used for determination of protein contents (Bradford, 1976), alkaline phosphatase activity (Babson, 1965),and acid phosphatase activity.,(Babson and Read 1959). Also, a 10 % homogenate of testes was prepared in ice-cold 0.1 M phosphate buffer, the homogenate was centrifuged at 12,000 xg for 30 min. at 4°C. The supernatant was used for determination of lactate dehydrogenase (LDH) activity (.Moss and Henderson (1994).

2.6 Statistical analysis

Data obtained were statistically analyzed using student's t-test at $p<0.05$ or less was considered significant (Petrie and Watson, 1999).

3. RESULTS & DISCUSSION

3.1 - Clinical observation and Mortality:

In the present chronic toxicity study, dicofol was administered into male rats at 4.19 amd 16.75 (ml/kg b.w), equal to 30 and 120 ppm respectively, in drinking water, for long-term exposure (90 weeks).

No visible signs of toxicity were noted during the experiment period, except emaciation (alopecia) and rough hair. The mortality was 30, 25, 13 % in the groups of rats dosed with higher and lower-dose and the control group, respectively.

3.2 – Effect of dicofol as Endocrine Disruptor:

3.2.1 – Effect on male fertility indices:

a) Testes and epididymus weight:

The testes of humans and other animals are highly susceptible to damage produced by genetic disorders, environmental or occupational exposure to chemicals or other means. Specific causes of testicular damage have been catalogued (Jackson and Ericsson, 1970; Jackson, 1973; Gomes, 1977).

Results demonstrated that dicofol at lower and higher-doses significantly decreased the weight of testes in all treated groups at week 16, 28 and 90 (Table 1). These changes were more marked at higher-dose and prolonged dicofol exposure. Meanwhile, decline in epididymus weight was observed only at the higher-dose level after 28 and 90 weeks (Table 1). In the present study, decline in testes weight was confirmed by the histopathological observations, since most of the somniferous tubules were degenerated and atrophied, as shown in Figures 1, 2, 3.

Similar results were recorded with O`Ch pesticides at different experimental period; i.e. DDT (Ben Rhouma et al., 2001), lindane (Chitra et al., 2001 and Sujatha et al., 2001) and endosulfan (Choudhary and Joshi, 2003).

Much data have been reported on the reproductive toxicity of chlorinated hydrocarbons and confirm our results. Brown and Casida (1987) and Jadaramkunti and Kaliwal (2002) showed that reduction of testes and epididymus weights in rats treated with the highest dose of dicofol for long-term are the result of reduction diameter of somniferous tubules, spermatogenic, Leydig and Sertoli cells.

On discussing the results with previous reports, it is proposed that, dicofol probably imped the activity of testes and epididymus by inhibition of androgen production, its antiandrogenic nature or its direct action on these organs (Kaur and Mangat, 1980). Moreover, the deleterious effects of dicofol on reproductive organ weights might be due to a decrease in testosterone T level after 16, 28 and 90 weeks from the onset of the treatment (Takizawa and Horii, 2002).

Several studies have shown that the epididymis and accessory sex organs require acontinuous androgenic stimulation for preservation of their normal structural and functional integrity (Mann, 1974). Thus, the slight reduction in the weight of the epididymis and accessory sex organs in the treated rats may be due to lower bioavailability of androgens (Mathur and Chattopadhyay, 1982).

Also, Sujatha et al. (2001) reported that, the decrease in testicular weight of lindane-treated rats (O`Ch) may be due to reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells. Furthermore, there is much concerns that exposure to estrogen –or estrogen-like chemicals induce major pathological effects in epididymus in men and experimental animals (Chitra et al., 2001). It could be concluded that dicofol may be acting on testes and accessory reproductive organs by blocking androgen biosynthesis and/or by antagonizing the action of androgens (Prasad and Vijayan, 1987). Also, the same authors mentioned that dicofol may be acting directly on the normal function of the hypothalamo-pituitary-gonadal axis.

b) Epididymal sperm count:

Sperm count is one of the most sensitive tests for spermatogenesis and it is highly correlated with fertility. Our results showed that, treatment of rats with dicofol at the lower and higher-dose levels for three durations; 16, 28 and 90 weeks, significantly, reduced the total sperm count in all treated groups (Table 1), the effect was dose and time dependent. Histological structure of the testes confirmed this; where it revealed degeneration and atrophy in some of somniferous tubules associated with low luminal spermatozoal concentration. These findings go hand in hand with those of Jandaramkunti and Kaliwal (2002) who found that the number of spermatogenic, spermatocytes, spermatides and Leydig cells were significantly decreased with higher-dose of dicofol and thus reduced sperm count. Also, the authors reported that quantity and quality of sperm production has been adversely affected following exposure of certain drugs and chemicals, particularly mutagens and teratogens. Furthermore, there is a clear correlation between the degree and duration of exposure to pesticides and the extent of spermatogenic arrest and hormonal imbalance.

c) Motility and viability of sperm:

The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperm are immotile, to distinguish between immotile dead sperm and immotile live sperm (Björndahl et al., 2003).

Our results revealed that, at week 16, after the administration of dicofol, a dose-dependent reduction in percentage of motile and live were observed. Also, at week 28 and 90, significant decrease was observed, the changes noted did not follow the expected dose-relationship. As regards the effect of time, dicofol has time dependent effect, however, not in uniform fashion (Table 1) and Figures (4, 5). Choudhary and Joshi (2003), also stated that, oral administration of rats with endosulfan (O`Ch) at doses of 5, 10 and 15 mg/kg b.w./day for 30 days, significantly, decreased the spermatozoal motility and density in cauda epididymus and testes in dose-dependent manner. Decline in sperm motility and density after oral administration of endosulfan is may be due to androgen insufficiency (Singh and Pandey, 1989; Chitra et al., 1999), which caused impairment in testicular functions by altering the activities of the enzymes responsible for spermatogenesis, this clearly suggests an antiandrogenic effect of endosulfan (Sinha et al., 1995 and Reuber, 1981).

d) Maturation of sperm:

From the aforementioned presentation, there were meaningful changes in the number of mature sperms in epididymus. Where, at week 16 and 28 results revealed marked decline in number of mature sperms, the differences seen were dose dependent. Furthermore, a time-related decreased in number of mature sperms was observed in dicofol-treated rats at the lower-dose; whereas, a trend (not significant) for this effect in dicofol-treated rats

at the higher dose (Table 1) and Figures (6,7). Morel et al., (1998) found a positive correlation between chromosomal abnormalities at the time of meiosis that cause disturbance during the transition of nucleoprotein and percentage of sperm nuclei that stained with aniline blue. The acidic aniline blue stains lysine-rich nucleoprotein of immature sperm. During spermatogenesis lysine rich histones are replaced by intermediate nucleoproteins which then are replaced by arginine and cysteine-rich protamines. Then, abnormal chromosome segregation at the time of meiosis allows the persistence of lysine-rich nucleoproteins in spermatozoa. It has been concluded that immature sperms were usually increased in infertile men (Moosani et al., 1994).

e) Sperm morphology:

Abnormal form percent was significantly increased in dose and time-dependent manner in all treated groups at the three durations (Table 1) and Figuress (8, 9, 10 and 11). This occurred as a result of toxic injury of dicofol to somniferous tubules as postulated from the histological examinations of testes in the treated animals.

Similar results were reported with certain organochlorine pesticides (O`C) at different experimental periods such as (i.e.), DDT (Ben Rhouma et al., 2001), lindane (Chitra et al., 2001 and Sujatha et al., 2001), dicofol (Jadaramkunti and Kaliwal, 2002), and endosulfan (Choudhary and Joshi, 2003).

Our results are in accordance with those of Tag El-Din et al. (2003), who reported that treatment with dicofol in higher dose (16.75 mg/ kg b.w./day, 5 days/week), for 6 months dosing period, significantly, reduced testes and epididymus weight, the total sperm count and percentage of motile and live sperms than lower dose (4.19 mg/kg b.w.). As well as, a significant increase in the percentage of abnormal forms was seen in higher-dose group, and this effect was dose-dependent. The authors noticed a marked depletion in number of mature sperm in the higher-dose group.

From the obtained results, it is interesting to notice that, dicofol seems to be more hazardous at higher dose and prolonged exposure period than lower dose and short exposure period, as revealed from its powerful effects on the weight of testes and epididymus or other semen parameters measured including; sperm motility, total sperm count, cauda epididymus sperm count, percentage of mature and live sperm, as well as abnormal forms.

Generally, the differences in fertility index data including statistical significant differences believed to dicofol effect. Notably, dicofol as well as, the administration periods played an important role in this respect, *i.e.* the effect was treatment and time dependent. The present study reveals that an exposure to dicofol may affect the histology of testes and sperm morphology. Accordingly, this testicular and spermatotoxic changes may be responsible for observed male mediated developmental toxic effects.

3.2.2- Effect on sex hormones:

Persistence of chlorinated insecticides and their congeners in the tissues of man and animals and in the environment post health problems of toxicological importance. One of these problems is the endocrinal dysfunction **(Rosiak *et al.*, 1997 and Hoekstra *et al.*, 2006**).

Recent evidence had suggested that organochlorine pesticides, even at low concentrations, may disrupt the endocrine system, which was responsible for proper hormone balance **(Mantovani, 2002 and Figa-Talamanca *et al.*, 2001)**.

a) Testosterone:

Testosterone T is the main steroid sex-hormone in male albino rats, it secreted by leydig cells of the testes under the control of complex neuroendocrine interactions **(Gornall and Goldbery, 1980; Robinson and Huntable, 1988)**.

In the present study, T level, significantly, decreased in all treated groups at lower and higher-doses at 16, 28 and 90 weeks of administration, except, in lower-dose group at 16 weeks, where T level unchanged after dicofol treatment (Table 2). Notably, there were no dose-related changes in T level, while a time-dependent reduction in higher-dose group was observed.

The significantly decrease of testosterone level, may be as a result of direct damage of dicofol on leydig cells, which are the main site of testicular androgen biosynthesis.

Results of the present work agree with those found by **Krause (1977), Desaulniers *et al.* (1999), Lafuente *et al.* (2000), Ben Rhouma *et al.* (2001)** and **Choudhary and Joshi (2003)**, who noted that T level was significantly decreased in male rats treated with organochlorine pesticides at different doses, *i.e.* DDT, PCB-126 and 153, methoxychlor, DDT, endosulfan, respectively.

Contrary to the results of the present investigation are those reported by **Foster *et al.* (1999)** and **Desaulniers *et al.* (1997)**, who mentioned that, rats administered with tris (4-chlorophenyl (TCPM), that is structurally related to DDT and dicofol, and PCB-28 (2, 4, 4`-trichlorobiphenyl) or PCB-77 (3. 3`, 4, 4`-tetrachlorobiphenyl) did not exhibit meaningful changes in T level.

b) Estradiol and progesterone:

Organochlorine pesticides have some estrogenic properties, and may modify the feed-back mechanism of steroids on the hypothalamus and pituitary **(Lafuente *et al.*, 2000).**

Exogenous estrogens (natural or synthetic) elicit all the pharmacologic responses usually produced by endogenous estrogens.

Estradiol (E_2) determinations have proved of value in a variety of contexts, including the investigation of precocious puberty in girls and gynecomastia in men **(March *et al.*, 1979).**

The present study also revealed that, significant, increase in E_2 level was observed in all dicofol-treated groups at the three durations (16, 28 and 90 weeks) of administration. In addition, dicofol significantly increased progesterone (P_4) level in treated groups; the effect was restricted only in duration of 16 and 28 weeks, and also there were no positive trend in this respect. (Table 2)

The present investigation in comparable to other (O`Ch) pesticides on account of exhibiting estrogenic activity of

dicofol when used in higher-dose for long-term **(Ball, 1984; Singh and Pandey, 1990; Ahlborg *et al.*, 1995; and Barton and Andersen, 1998)**.

The significant elevation of steroids for example, P_4 and E_2 in male rats which received lower and higher-doses of dicofol for long-term exposure could be attributed to increase the incidence of hypertrophy and/or vacuolation (empty cavities) of the adrenal cortex that enhanced the steroidogenic activity **(Solomon and Kulwish, 1991; Lindane, 1999)**. Moreover, **Jadaramkunti and Kaliwal (2002)** and **Tag El-Din *et al.* (2003)** suggested that dicofol mimic estrogenic activity when compared to other chlorinated pesticides (O`Ch) which may have a direct effect on the testes or indirectly through the hypothalmo-hypophyseal testicular axis or by desensitizing the testes to gonadotropins.

Furthermore, it has been reported that, the estrogenic like effects may be produced as a result that dicofol binds to estrogen receptors and exhibits estrogenic activity **(Stephen, 2001),** or by direct effect on sertoli cells resulting decreased FSH receptor binding and decreased 3-hydroxy-steroid-dehydrogenase activity that change estradiol to androgen, thus raising estradiol levels **(Wiebe *et al.*, 1983 and Colborn *et al.*, 1993).**

These findings are in close agreement with those reported by **Tag El-Din *et al.* (2003)**, who stated that dicofol at two doses 4.19 and 16.75 mg/kg b.w./day, in drinking water, for 6 months increased E_2 and P_4 levels in male rats in a dose-dependent manner.

Many pesticides are able to block or activate the steroid hormone receptors and/or to affect the levels of sex hormones, thereby potentially affecting the development or expression of the male and female reproductive system or both. This emphasizes the relevance of screening pesticides for a wide range of hormone-mimicking effects **(Vinggaard *et al.*, 2000).**

3.2.3 Effect on reproductive hormones:

a) Luteinizing hormone and follicle-stimulating hormone:

Luteinizing hormone (LH) is glycoprotein released from the anterior pituitary; it stimulates T production by leydig cells of the testes in males. Hypothalamic control of LH appears to be by a common releasing hormone termed gonadoliberin (GnRH, LHRH), with negative feedback control at the hypothalamic level by E_2 in the female and T in the male **(Gornall and Goldbery, 1980).**

Our results revealed that, short-term dicofol exposure (16 weeks) did not exert appreciable changes in LH and FSH levels in both lower and higher-dose groups. On the contrary, prolonged dicofol-exposure (28 and 90 weeks) significantly decreased LH and FSH levels in dose and time-dependent manner (Table 2).

These results go hand in hand with those of **Tag El-Din *et al.* (2003)** who reported that dicofol at 4.19 and 16.75 mg/kg b.w./day, for 6 months, induced significant decrease in LH level in male rats.

According to the suggestion reported by **Singh and Pandey (1990)**, the changes in the pattern of the steroidogenic enzymes 3 ß-hydroxysteroid dehydrogenase and 17 ß-hydroxysteroid dehydrogenase lead to inhibition of testicular androgen biosynthesis in adult rats, which is required for spermatogenesis in seminiferous tubules and sperm maturation in the epididymus. A complementary proposed mechanism, could explain dicofol induced toxicity, is blocking gonadotropin production and/or release by the pituitary, thereby testosterone production by leydig cells is not stimulated, causing spermatogenesis arrest **(Vanage *et al.*, 1997).** This mechanism is supported by the data previously reported by **Mably *et al.* (1992)** who recorded that, the alteration of LH had led to destruction of seminiferous epithelium and loss of germinal elements results in the reduction of the number of spermatids, sperm production in the testes as well as increase abnormality of sperms.

Marked decline in LH and FSH levels in the present study confirm the findings of **Desaulniers *et al.* (1999)** and **Lafuente *et al.* (2000)**, who investigated the toxicological influences of PCB (126 and 153) and methoxychlor, O`Ch, at different concentrations on male rats. On the other hand, the results of the present study disagree with the findings of **Tag El-Din *et al.* (2003)**, who mentioned that FSH level increased significantly after treatment with dicofol at lower and higher-doses (4.19 and 16.75 mg/kg b.w./day), in drinking water, for 6 months.

It was proved that certain O`Ch pesticides did not alter LH and FSH levels when administered into rats at different doses for short-term intervals, such as : TCPM **(Foster *et al.*, 1999)**, DDT **(Krause, 1977)** and PCB 28 and 77 **(Desaulniers *et al.*, 1997).**

An elevation in circulating levels of inhibin, a glycoprotein of primarily sertoli cell origin which inhibits FSH synthesis and secretion by the pituitary **(Caroll *et al.*, 1991)**, could account for the observed decrease in serum FSH level in the current study which was confirmed histopathologically by degeneration and atrophy of seminiferous including leydig and sertoli cells. FSH stimulates the sertoli cells of the seminiferous tubules to produce androgen binding protein, probably moves via the sertoli cells to other germ cells and to the epididymus where the testosterone is released to exert its physiological effects in sperm maturation **(Mably *et al.*, 1992).**

3.2.4 – Effect on thyroid hormones:

a) Tri-iodothyronine and thyroxine:

The disruption of thyroid hormone homeostasis by a variety of xenobiotics has been associated with thyroid follicular cell hypertrophy, hyperplasia, and the development of thyroid tumors in rats **(Hill *et al.*, 1989 and Capen, 1996)**. Thyroid toxicants affect circulating levels of thyroid hormone by either direct action on the thyroid gland or by increasing peripheral elimination of thyroid hormone **(Capen, 1996)**.

Concerning the thyroid hormones; dicofol, at lower and higher-doses, induced significant decrease in thyroxine (T_4) and tri-iodothyronine (T_3) levels throughout the experimental periods (16, 28 and 90 weeks), except at 16 weeks T_4 level did not alter significantly in lower-dose

group (Table 2). These changes were more marked at higher dose of dicofol as well as, prolonged dicofol exposure. It is worth to say, results revealed evident affection of thyroid gland, such affection was dose and time-dependent.

Hypothyrodism significantly reduced seminiferous tubule and lumen diameter, where in hypothyroid rats, the proliferation and differentiation of germ cells were arrested and their number was decreased **(Maran and Aruldhas, 2002)**, the present study clearly indicates that hypothyrodism adversely affects spermatogenesis; it also indicates that thyroid hormones are essential for normal spermatogenesis.

In accordance with the findings of the present study, **El-Kashoury *et al.* (2003)** described similar changes in T_4 and T_3 levels after dicofol exposure at lower and higher-doses. They also mentioned that the decrease in T_4 levels may be a result of iodine deficiency, the gland fails to synthesize T4 and hypothyrodism occurs. Another suggestion reported by **Hotz *et al.* (1997)** who reported that, pesticide increased deiodination and biliary excretion of thyroid hormone T_4 which led to increased rate of T_4 elimination from the blood.

A complementary proposed mechanism, could explain organochlorine (O`Ch) induced toxicity, is attributed to their ability to deplete stores of vitamin A and thyroid hormones from the body by 30-50 %, through interaction with a common plasma protein carrier called transthyretin (TTRs) and alteration of their metabolism in the liver and other organs. T_4 and Vit. A are known to be important regulators of normal epithelial differentiation and proliferation **(Heussen *et al.*, 1993)**. Another support to the interaction of O`Ch with TTRs was established by **Van den Bery *et al.* (1991)** who mentioned that hydroxylated PCBs and number of halogenated industrial chemicals, mainly pesticides (O`Ch) may decrease thyroid hormone levels in rats through interference with hormone transport carriers (TTRs).

An alteration in thyroid hormone T_4 and/or T_3 level in the present study confirm the findings of **Desaulniers *et al.* (1997). Desaulniers *et al.* (1999) and Fisher *et al.* (2006)**, who investigated the toxicological influences of certain organochlorine pesticides "O`Ch" at different concentrations, *i.e.*, PCB-28 (2, 4, 4`-trichlorobiphenyl) and PcB-126 (3, 3`, 4, 4`, 5-pentachlorobiphenyl).

Another mechanism was postulated by **Villa *et al.* (2004)** who stated that "O`Ch" might alter the expression of a membrane of genes by a direct receptor mechanism. This receptor is made of a basic protein and known as aryl hydrocarbon receptor (AHR) which is maintained in a ligand binding state in association with cytosolic protein **(Martinez *et al.*, 2002).** Exposure to "O`Ch" and related compounds leads to dissociation of AHR from the binding protein **(Lund *et al.*, 1988)**, which is transferred to the nucleus and then it binds to specific DNA leading to severe harmful effects such as induction of cytochrome P_{450} (CYP_{450}) 1A1 gene **(Dacroix and Hantella, 2003)**. Lastly, bioactivation of these compounds can make them more toxic which may modulate the expression of the related genes in the tissues **(Mansour, 2004).**

3.3 - Effect on Oxidative Parameters:

Pesticides and environmental chemicals may induce oxidative stress leading to generation of free radicals and alteration in antioxidants or oxygen free radical (OFR) scavenging enzyme system **(Ahmed *et al.*, 2000)**.

3.3.1 Lipid peroxidation :

Generation of oxidative stress and consequent lipid peroxidation (LPO) by pesticides is reported in many species. It has been reported that increase in ROS can cause the destruction of all cellular structures including membrane lipid **(Ichikawa *et al.*, 1999)**. Hence in the present study, lipid peroxidation is used as an index of oxidative stress. Several drugs, xenobiotics and environmental pollutants are known to cause imbalance between formation and removal of free radicals **(Verma *et al.*, 2007)**.

ROS such as superoxide anions ($O^{\cdot}_2$), hydroxyl radical ($O^{\cdot}H$) and H_2O_2 enhance oxidative process and produce lipid peroxidative damage to cell membranes. The ($O^{\cdot}H$) radical has been proposed as an initiator of LPO through an iron-catalysed Fenton reaction **(Kale *et al.*, 1999).** LPO is the process of oxidative degeneration of polyunsaturated fatty acid (PUFA) and its occurrence in biological membranes causes impaired membrane function, structural integrity **(Gutteridge and Halliwell, 2000)**, decrease in membrane fluidity and inactivation of a several membrane bound enzymes.

Results in (table 3) showed that, an administration of rats with dicofol, at lower and higher doses for two durations 28 and 90 weeks, increased oxidative stress in cauda epididymus of rats, as evidenced by enhanced levels of malondialdehyde (MDA) level.

An elevation of LPO in cauda epididymus, as evidenced by increased production of MDA in the present study, suggests participation of free radical-induced oxidative cell injury in mediating the toxicity of dicofol. These intentionally introduced environmental xenobiotics are known to have a strong affinity for interaction with membrane phospholipids **(Sharma *et al.*, 2005)**. An elevation in LPO caused by other O`Ch in different experiments has also been reported; methoxychlor **(Latchoumycandane and Mathur, 2002a)**, methoxychlor (**Latchoumycandane and Mathur, 2002b)**, endosulfan **(Kwon *et al.*, 2005)** and 2, 3, 7, 8-tetrachlorobenzo-P-dioxin (TCDD) **(Latchoumycandane *et al.*, 2003)**.

3.3.2 Glutathione level:

Glutathione (GSH) one of the most abundant antioxidant in cells has been found to decrease during apoptosis. GSH has been hypothesized to play a role in the rescue of cells from apoptosis, by buffering an endogenously induced oxidative stress **(Fernandez *et al.*, 1995)**.

In our study, a decrease in GSH levels in dicofol-intoxicated animals may be responsible for enhanced LPO **(Younes and Siegers, 1981 and Goel *et al.*, 2005)**. Our results confirm the findings of **Tithof *et al.* (2000); Selzak**

et al. **(2000); Luna Samanta (2002); Latchoumycandane *et al.* (2002a)** and **Saradha and Mathur (2006)**, who reported that repeated administration of several organochlorine at different concentrations (dicofol, 2, 3, 7, 8-tetrachlorodibenzo-P-dioxin (TCDD), hexachlorocyclohexane (HCH), methoxychlor and lindane, respectively) induced disturbances in the activities of the enzymes regulating GSH metabolism.

As regards GSH level and lipid peroxidation in normal rats (control group), it is worth to mention that, there were significant differences between the control groups (28 and 90 weeks). A noticeable decrease in GSH level accompanied by concomitant increase in LPO in 90 weeks compared with their levels in 28 weeks was observed. It is well documented that advancing age an organism is under greater oxidative stress as the result of impairment of the function of mitochondrial respiratory chain **(Wei and Lee, 2001)**. This leads to an accumulation of DNA, RNA and protein free radical damage **(Holmes *et al.*, 1992)** and causes alterations in antioxidant enzyme levels **(Sanz *et al.*, 1997)**.

3.4 -Effect on Testicular Functions:

Organochlorine pesticides (O`Ch), having estrogenic property, impaired the testicular functions through altering the testicular biochemistry **(Sinha *et al.*, 1995; Chitra *et al.*, 1999 and Choudhary and Joshi, 2003)**.

3.4.1 Total protein:

The testicular fluid contains both stimulatory factors as well as inhibitory factors that selectivity alters the protein secretions **(Brooks, 1983)**. Thus, the changes in protein suggested that there is a reduction in the synthetic activity in testes.

From Table 4, it is clear that, an administration of dicofol into rats leads to elevation of protein concentration in testes at two doses (lower and higher). Except, at higher-dose for 28 weeks, no appreciable changes in protein content were observed.

Similar elevation in protein content caused by other "O`Ch" has also been reported **(Shivanandapp and Krishnakumari, 1981; Bhatnagar and Malviya, 1986)**.

The accumulation of protein occurred in testes and epididymus due to androgen deprivation to target organs. This deprivation effect also led to a reduction in testicular and cauda epididymal sperm population, loss of motility in the latter and an increase in number of abnormal spermatozoa, thereby manifesting 100 % failure in fertility in treated animals **(Rao and Chinoy, 1983)**.

Concerning the protein content, our findings, are in accordance with those reported by **El-Kashoury and Mansour (2007)** who studied the effect of dicofol at two doses, for long term, on testicular biochemistry.

3.4.2 Acid and alkaline phosphatase (ACP & ALP) :

Acid phosphatases are enzymes capable of hydrolyzing orthophosphoric acid esters in an acid medium. The testicular acid phosphatase gene is up-regulated by androgens and is down-regulated by estrogens **(Yousef *et al.*, 2001)**. Activities of free lysosomal enzymes have been shown to rise when testicular steroidogenesis is increased **(Mathur and Chattopadhyay, 1982)**.

Based on the data obtained in this study, dicofol when administered into rats, at lower and higher doses for different duration (28 and 90 weeks) induced significant decrease in ALP activity (Table 4). It is clear that, the changes noted did not follow the expected dose and/or time relationship. A decrease in ALP activity indicated that dicofol treatment produced a state of decreased steroidogenesis where the inter- and intracellular transport was reduced as the metabolic reactions to channalize the necessary inputs for steroidogenesis slowed down **(Latchoumycandane *et al.*, 1997)**.

As regards ACP enzyme, results of the present study showed significant decrease in its activity during two durations (28 and 90 weeks). While, there were no treatment-related changes, but a time-dependent effect in higher-dose groups was detected.

As regards ALP and ACP activities, results of the present investigation were similar to those reported by **Chitra *et al.* (1999)**. A decrease in the ACP in Free State would thus reflect decreased testicular steroidogenesis in rats and this may be correlated with the reduced secretion of gonadotrophins **(Latchoumycandane *et al.*, 1997).**

3.4.3 Lactate dehydrogenase (LDH) :

Testicular LDH is an essential component of the metabolic machinary of spermatozoa and involved in the energy generation processes.

An administration of rats with dicofol at both doses (lower and higher) decreased significantly LDH activity in testicular tissues. All changes in LDH activity mean that no dose relationship. On the other hand, the higher-dose exhibited time-dependent effect (Table 4). The decreased in LDH activity in dicofol-treated rats points toward the interference of dicofol with the energy metabolism in testicular tissues **(Mollenhauer *et al.*, 1990)**.

The correlation between LDH and motility and living sperm could be a sign that extracellular LDH ensures metabolism of spermatozoa, perhaps even in anaerobic conditions. This hypothesis is underlined by the significant negative correlation between LDH and pathomorphology of sperm **(Kamp *et al.*, 1996)**.

In the present study, male fertility indices were measured confirm the above-mentioned suggestion where, marked declined in count, motility and viability of sperm were observed as well as an elevation in abnormalities of sperm. It means that LDH enzyme has an important role in the normal energy supply in spermatogenesis.

Notably, decline in serum T level was observed with a reduced reproductive organ weights, which means that male reproductive toxicity induced by dicofol would be augmented by decreased serum T level as well as a decreased function of sertoli and leydig cells, in addition to the direct cytotoxic effect on germ cells **(Takizawa and Horii, 2002)**.

In view of this data, it can be concluded that dicofol induced disorders of reproductive system result from a

disturbance of the androgen-estrogen balance, as well as oxidative stress and impairment in testicular functions. Although, it is not possible link all these events together, it is assumed that their collective impact to ultimately leads to a perceptible change in sex hormone balance and arrest of spermatogenesis. Further studies are need for better understanding of the cause of reproductive toxicity induction of dicofol, and possibly of o'ch as a whole.

Table (1): Influence of Dicofol on testes, epididymus weight and semen parameters after chronic exposure in drinking water, for 90 weeks.

Periods	16 Weeks			28 Weeks			90 Weeks		
Parameters	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Testes weight (g)	1.55 ±0.035	1.35 ±0.016*** a	1.30 ±0.071* a	1.58 ±0.034	1.50 ±0.041	1.38 ±0.051* a	1.95 ±0.011	1.48 ±0.137*** a	1.19 ±0.092*** a
Epididymus weight (g)	0.20 ±0.007	0.19 ±0.011	0.18 ±0.010	0.35 ±0.020	0.30 ±0.019	0.25 ±0.014** a	0.47 ±0.005	0.39 ±0.037 **b	0.24 ±0.025*** a
Sperm count	100.0 ±3.536	90.0 ±2.236** a ***b	60.0 ±3.162*** a	90.0 ±2.915	35.0 ±3.536*** a	30.0 ±2.550*** a	110.0 ±3.742	20.0 ±3.536*** a	15.0 ±3.536*** a
Motility (%)	90.0 ±1.581	65.0 ±3.536** a **b	40.0 ±3.536*** a	85.0 ±3.317	20.0 ±2.915*** a	15.0 ±2.550*** a	85.0 ±2.449	35.0 ±8.367*** a	25.0 ±8.660*** a
Viability (%)	90 ±2.236	70 ±2.550*** a **b	50 ±3.536*** a	90 ±2.550	40 ±3.536*** a	30 ±3.317*** a	90 ±1.225	40 ±3.742*** a	35 ±6.708*** a
Mature sperm (%)	90 ±2.550	80 ±3.536 **b	60 ±3.536*** a	80 ±3.536	65 ±2.550*** * b	50 ±5.100** a	90 ±1.225	55 ±5.099*** a	50 ±6.403*** a
Abnormal forms (%)	15 ±2.236	25 ±3.536** a **b	40 ±1.871*** a	20 ±1.871	35 ±3.536*** a ***b	65 ±3.536*** a	20 ±1.225	65 ±2.236*** a **b	50 ±3.742*** a

Values represent means ± SE, n = 5 * P <0.05 * * P <0.01 * * * P <0.001 (Student's t-test)
a: treated group versus control group b: lower dose group versus higher dose group

Table (2): Influence of dicofol on sex steroid, reproductive and thyroid hormones after chronic exposure in drinking water for 90 weeks

Periods	16 Weeks			28 Weeks			90 Weeks		
Parameters	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Testosterone (ng/ml)	1.30 ±0.230	1.10 ±0.311	0.60 ±0.141 * a	3.60 ±0.311	1.40 ±0.270*** a	1.30 ±0.230*** a	2.10 ±0.241	1.30 ±0.230* a	1.20 ±0.270* a
Estradiol (Pg/ml)	9.40 ±0.540	23.0 ±0.707*** a ** b	17.00 ±1.414 ** a	14.80 ±0.354	21.70 ±0.212*** a * b	23.80 ±0.707*** a	8.70 ±0.283	13.20 ±0.270*** a *** b	9.90 ±0.396* a
Progesterone (ng/ml)	11.63 ±0.396	18.42 ±0.544*** a	18.89 ±0.652*** a	7.23 ±0.326	9.00 ±0.707 * b	11.44 ±0.439*** a	4.84 ±0.334	4.97 ±0.369	6.00 ±0.500
FSH (ng/ml)	2.00 ±0.354	1.40 ±0.070	1.20 ±0.141	2.50 ±0.354	1.90 ±0.192 *** b	0.30 ±0.071*** a	3.20 ±0.184	0.80 ±0.071*** a *** b	0.25 ±0.050*** a
LH (ng/ml)	3.53 ±0.212	3.10 ±0.283	2.90 ±0.241	3.20 ±0.184	1.34 ±0.114*** a	1.18 ±0.141*** a	2.90 ±0.200	0.97 ±0.130*** a	1.40 ±0.184*** a
Thyroxine (ng/ml)	26.88 ±3.942	18.8 ±2.628	14.45 ±0.987* a	29.37 ±1.170	21.00 ±1.043*** a *** b	14.41 ±0.544*** a	43.83 ±0.472	37.82 ±2.381* a *** b	20.02 ±2.038*** a
Tri-iodothyrine (ng/ml)	0.46 ±0.046	0.32 ±0.029* a	0.27 ±0.012** a	0.41 ±0.012	0.36 ±0.016 * a * b	0.29 ±0.019*** a	0.33 ±0.014	0.28 ±0.016* a *** b	0.16 ±0.003*** a

Values represent means ± SE, n = 5 * P <0.05 * * P <0.01 * * * P <0.001 (Student's t-test)
a: treated group versus control group b: lower dose group versus higher dose group

Table (3): Influence of Dicofol on oxidative parameters in cauda epididymus and testicular functions:

Periods	28 Weeks			90 Weeks		
Parameters	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Malondialdehyde (µmol /g tissue)	93.07 ±1.516	146.84 ±11.048** a	166.76 ±14.210** a	181.44 ±9.610	219.75 ±8.740* a	199.69 ±7.814
Total glutathione (µmol /g tissue)	374.26 ±1.692	280.36 ±3.382*** a *** b	321.91 ±4.368*** a	291.41 ±5.074	154.69 ±4.227 *** a *** b	220.98 ±2.537*** a
Total protein (mg/g tissue)	17.90 ±0.511	22.09 ±0.371** a ** b	18.19 ±0.686	16.57 ±0.399	20.41 ±1.220* a	20.14 ±0.621** a
Alkaline phosphates (U/mg protein)	0.085 ±0.007	0.054 ±0.001** a	0.057 ±0.003* a	0.094 ±0.003	0.070 ±0.004** a	0.074 ±0.005* a
Acid phosphates (U/mg protein)	0.112 ±0.002	0.084 ±0.003*** a * b	0.099 ±0.004* a	0.125 ±0.003	0.089 ±0.004*** a	0.084 ±0.004*** a
Lactate dehydrogenase (U/mg protein)	1.55 ±0.043	1.11 ±0.046*** a *** b	1.53 ±0.036	1.60 ±0.034	1.28 ±0.090* a	1.33 ±0.045** a

Values represent means ± SE, n = 5 * P <0.05 * * P <0.01 * * * P <0.001 (Student's t-test)
a: treated group versus control group b: lower dose group versus higher dose group

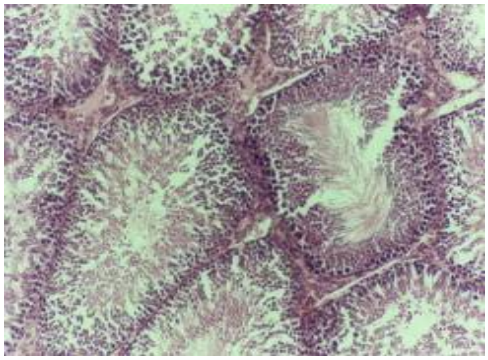

Figure (1): light photomicrograph of a section of the Testes of rats in control group, showing the normal mature seminiferous tubules with complete series of spermatogenesis and high spermatozoal concentration in the lumen (H & E X40)

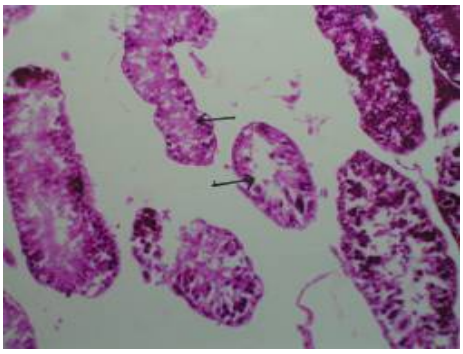

Figure (2): light photomicrograph of a section of the Testes of rats administrated 30 ppm of dicofol (lowere dose) for 28 weeks, showing degeneration and atrophy of some seminiferous tubules (arrow) (H & E X40)

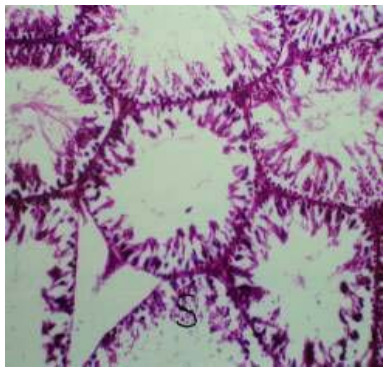

Figure (3): light photomicrograph of a section of the Testes of rats administrated 120 ppm of dicofol (higher dose) for 16 weeks, showing degeneration of the seminiferous tubules (S) with depression in lumenal spermatozoal concentration (H & E X40)

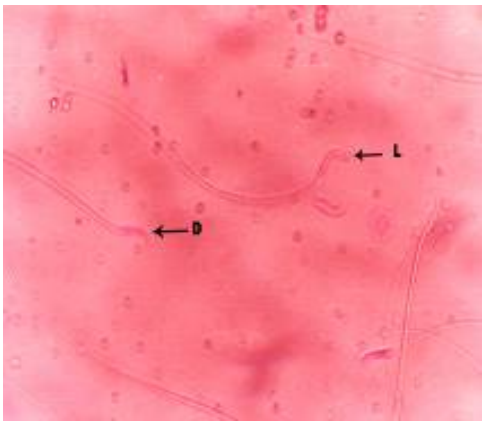

Figure (4) Showed live unstained sperm (L) and dead sperm (D) stained with eosin stain in rats treated with dicofol in drinking water (40X)

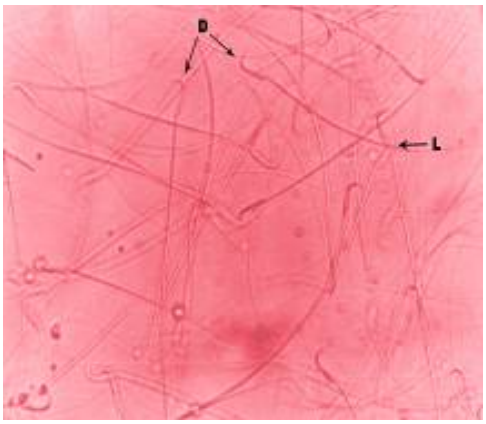

Figure (5) Showed live unstained sperm (L) and dead sperm (D) stained with eosin stain in rats treated with dicofol in drinking water (40X)

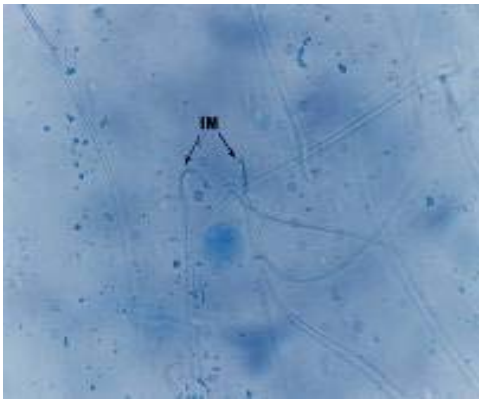

Figure (6) Showed nuclear mature sperm (M) was not stained and immature sperm (IM) with abnormal chromosomes stained with aniline blue in rats treated with dicofol in drinking water (40X)

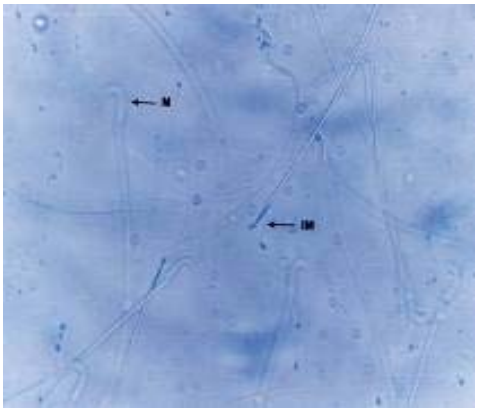

Figure (7) Showed immature sperm (IM) stained with aniline blue color in rats treated with dicofol in drinking water (40X)

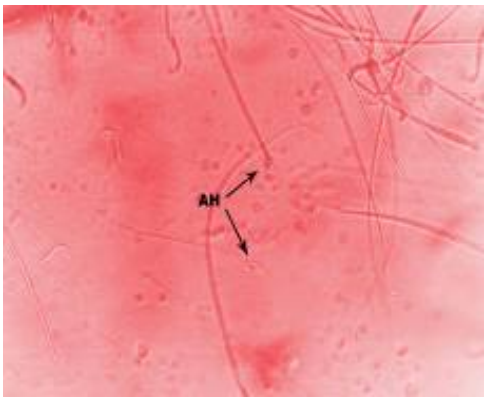

Figure (8) Showed abnormal head sperm miss-shape (AH) stained with eosin stain in rats treated with dicofol in drinking water (40X)

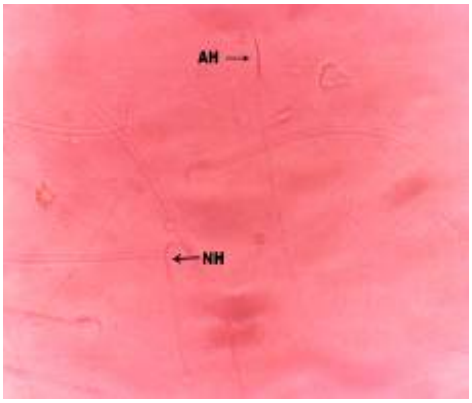

Figure (9) Showed normal sperm with normal head hook shape (NH) and abnormal head sperm no hook (AH) in rats treated with dicofol in drinking water (40X)

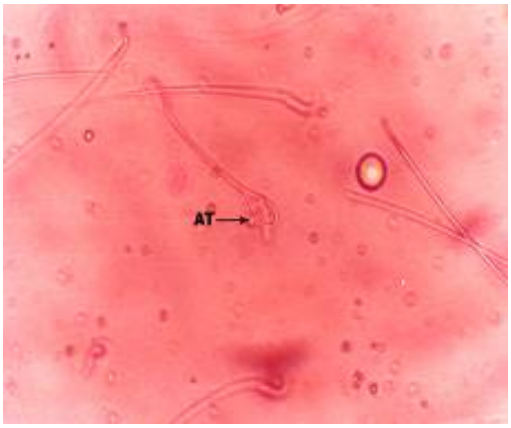

Figure (10) Showed bent tail tip sperm (AT) stained with eosin stain in rats treated with dicofol in drinking water (40X)

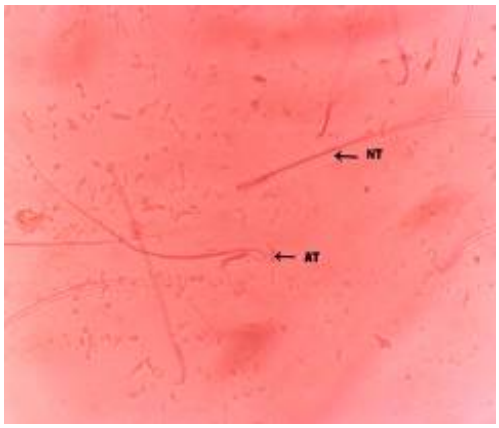

Figure (11) Showed normal tail sperm (NT) and bent tail sperm (AT) in rats treated with dicofol in drinking water (40X)

ABBREVIATIONS USED:

O`Ch, Organochlorine insecticides; ROS, Reactive oxygen species; PUFA, Polyunsaturated fatty acid; EC, Emulsifiable concentrate; T_4, Thyroxine; T_3, Triiodothyronine; T, Testosterone; P_4, Progesterone; E_2, Estradiol; FSH, Follicl-Stimulating hormone; LH, Luteinizinghormone; ALP, AlkalinePhosphatase; ACP, Acid Phosphatase; LDH, Lactate Dehydrogenase; LPO, Lipid Peroxidation; GSH, Glutathione.

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Exploring Biotechnology For Conserving Himalayan Biodiversity

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Abstract: The Himalaya is one of the largest and youngest mountain ranges of the world, and covers 10 percent of India's land area. Extending across much of the northern and northeastern borders of the country, the Himalayan massif regulates climate for a broad portion of Asia and provides ecosystem services (especially perennial water systems) to much of the heavily populated plains of India. The outlook for the future of bioprospecting and biodiversity conservation is difficult to predict, but the fact that the issue remains at the forefront of current debate, and that there are ongoing developments on both the institutional and economic front, suggests that much work remains to be done. There is a growing realization of the need for a clearer institutional framework, and for better involvement of local communities, but until concrete steps are taken in this direction, success stories will remain scattered. Under ideal conditions, bioprospecting can be an effective way to preserve biodiversity locally, and it can play an effective, albeit limited role, in overall efforts to conserve global biodiversity. [Life Science Journal 2010;7(3):20-28]. (ISSN: 1097-8135).

Keywords: Himalaya; Asia; bioprospecting; biodiversity; conservation; global

"*Biodiversity is the very core of our existence within our communities. You cannot say how many dollars this is worth because it is our culture and our survival. In this context biodiversity is invaluable … We value our surroundings as our identity, as who we are and our inheritance that is given to us … Our environment is many things, a classroom, a pharmacy, and a supermarket.*" **Ruth Lilongula, Solomon Islands (UNEP/IT, 1999, p.162)**

Himalayan Biodiversity

The Himalaya is one of the largest and youngest mountain ranges of the world, and covers 10 percent of India's land area. Extending across much of the northern and northeastern borders of the country, the Himalayan massif regulates climate for a broad portion of Asia and provides ecosystem services (especially perennial water systems) to much of the heavily populated plains of India. In addition, due to its unique location as the meeting place of three biogeographic realms (the Palaearctic, Indo-Malayan and Mediterranean), species diversity and endemism in the region is unique. At the same time the region is extremely fragile as a complex result of tectonic activities and anthropogenic influences. On account of its unique and diverse ecosystems and high levels of threat, the Himalaya has been recently designated as a global biodiversity hotspot by Conservation International.

In northern India, the Himalaya extends across the states of Jammu and Kashmir, Himachal Pradesh and Uttaranchal. The Himalayan region falling within this zone is classified into two major biogeographic zones: the Trans-Himalaya and the Himalaya. The windward slopes of the Great Himalaya and associated ranges form a large biophysical zone is classified under the Himalaya biogeographic zone **(Rodgers and Panwar 1988)**. Ecosystems in this zone encompass one of the largest altitudinal gradients in the world, range from the subtropical forests of the Siwaliks to alpine meadows and scrub in the higher peaks of Great Himalayas. Some of the richer assemblages of wild and medicinal plants are found in this region. It has been estimated that the region supports over 4500 species of vascular plants **(Western Himalaya Ecoregional BSAP 2002)**. **Champion and Seth (1968)** classification includes 11 major types (and 47 subtypes including several stages and disturbance types) in the Himalaya. The key features of biological diversity in this region include: i) wide latitudinal, altitudinal and moisture gradients encompassing a large number of ecosystem types, ii) high levels of diversity and endemism, iii) unique examples of agro-biodiversity, iv) species of great commercial value, and v) unique indigenous knowledge systems.

Agro-biodiversity recorded from the region is unique and records of medicinal plant species are available which are traditionally being used by the people. The mid-elevation oak (*Quercus spp*.) forests found in the region are ecologically as well as economically important. A number of species such as *sal* (*Shorea robusta*), *chir* pine (*Pinus roxburghii*) and *deodar* (*Cedrus deodara*) has been extracted for their wood. Recently extensive harvesting of medicinal species such as *Taxus wallichiana, Aconitum heterophyllum* and *Picrorrhiza kurrooa* is causing concern. High plant species diversity and productivity of this zone is matched by a diverse assemblage of faunal elements. Avifauna in this region is diverse and over 640 species of birds have been reported of which 205 are endemic **(Western Himalaya Ecoregional BSAP 2002).** Bird species of maximum conservation

importance include the pheasants such as the Western Tragopan (*Tragopan melanocephalus*), the satyr tragopan (*Tragopan satyra*) and the Cheer pheasant (*Catreus wallichi*). With respect to mammals, the lower altitudes, especially the Siwalik zone has significant populations of elephant (*Elephas maximus*) and tiger (*Panthera tigris*). The temperate zone has a large number of resident species; among these are endangered species like the musk deer (*Moschus chrysogaster*), the Himalayan *tahr* (*Hemitragus jemlahicus*) and the Kashmir stag or *hangul* (*Cervus elaphus hangulu*). Compared to birds and mammals, reptiles and amphibians are less studied and less diverse, especially in the higher altitudes. Fish species diversity is considerable and a large number of fish species have been introduced into the region. The golden *mahseer* (*Tor*), which is found in the lower and middle altitude streams and rivers, is now endangered. Reliable estimates of invertebrate diversity for the region are not available. Over 450 species of butterflies (*Lepidoptera*), more than (each) of *Hemiptera* and *Isoptera* are reported from the region **(Western Himalaya Ecoregional BSAP 2002).**

The impact of biotechnology on various aspects and economic progress of various nations around the world has given a major impetus to accelerate research, development and application of this field in relevant socio-economic sectors. Himalayan biodiversity is a woderful niche for exploring the potential of microbial, animal and plant world. The cell fusion techniques, recombinant DNA technology, protein engineering and structural biology have made phenomenal progress as priority research areas. In addition to basic research, the scientists are actively engaged in fermentation based activities, production of valuable biologicals, plant or animal cell culture, marker assisted selection and breeding, value addition, prospecting of biological resources, molecular taxonomy and micropropagation methods for producing high quality, genetically superior planting materials.

Present Problem

The primary threats to biodiversity conservation in the Himalaya include deforestation, commercial extraction of medicinal plants, grazing, invasive species, poaching, and growth of orchards, pollution, eutrophication and global warming. Over the last decade, the high rates of biodiversity loss, particularly in developing countries, have come to the forefront as one of the two most urgent global environmental issues. At the same time, the biotechnology industry has grown rapidly, and the two issues have become closely linked.

The Convention on biological diversity started as a document drawn up by IUCN on the *in situ* conservation of biodiversity. The document was submitted to the UNEP Governing Council, which accepted the need for an international biodiversity convention and accepted responsibility for its drafting. The draft convention was broader than the IUCN document and covered conservation, wild species of commercial crops, and the transfer of technology, biotechnology and expertise to developing countries. Formal negotiations, involving different delegates from 75 countries, started in November 1990 and a final version of the convention was signed in 1992 by 156 nations (including Pakistan) at the UN Conference on Environment and Development, the Earth Summit, in Rio de Janeiro. The convention aims to save animal and plant species from extinction and restore their habitats.

The convention stipulates that parties must develop national strategies for the conservation and sustainable use of biological resources; establish protected are as, resuscitate degraded ecosystems, control alien species and establish conservation facilities; establish training and research programmes for the conservation and sustainable use of biodiversity and support such programmes in developing countries; promote public education and awareness regarding conservation and sustainable use of biodiversity; carry out an environment impact assessment prior to any proposed project that may reduce biodiversity; recognize the right of governments to regulate access Dragon fly/Khushal Habibi Cobra/Ayesha Vellani *Calotropis procera*/WWF-Pakistan to their own genetic resources, and wherever possible, grant other parties access to genetic resources for environmentally sound uses; encourage technology and biotechnology transfer, particularly to developing countries; establish an information exchange between the parties on all subjects relevant to biodiversity; promote technical and scientific cooperation between parties, particularly between developing countries, to enable them to implement the convention; ensure that countries that provide genetic resources have access to the benefits arising from them; and, provide financial resources to developing countries in order to enable them to carry out the requirements of the Convention.

OVERVIEW OF BIOTECHNOLOGY AND BIODIVERSITY CONSERVATION

Strengths and Status

The Himalaya a global hotspots of biodiversity, is now receiving importance from researchers as well as policy makers and a number of institutions are involved in biodiversity conservation in the region. A few of these are solely focused on the Himalaya, and the region also benefits from a number of national level state of the art institutions and scientific expertise that is located within the region. Significant strides in biodiversity research have been made by universities and institutes located in the region. Although yet to be implemented at the grass-roots level, sustainable use models, traditional livelihood practices, knowledge and benefit-sharing are finding mention in recent policies and planning documents. These prepare the ground for future initiatives relating to participatory conservation and sustainable use frameworks.

Currently, a number of NGO initiated livelihood and sustainable use projects are going on. One of the unique capacities of the region is the heightened environmental consciousness of local communities. This is especially so in the Uttarakhand Himalaya where voluntary movements to protect forests and biodiversity have been initiated by the local people.

Priorities and Strategies

The need of the hour is to conserve biodiversity through physiological and biotechnological advancements. In particular, it is to determine whether the biotechnology industry, through bioprospecting, can generate enough socially sustainable profits to function as an incentive to biodiversity conservation. After an overview of the links between biodiversity conservation and biotechnology and their early history, present goal is to analyze the current institutional framework surrounding this issue, and in particular the conflict between the TRIPs regime and the Convention on Biological Diversity over property rights on genetic resources and traditional knowledge. Additionally, need is to look at whether bioprospecting efforts have achieved enough economic viability – in terms of profits generated for the private sector – and social sustainability – in terms of benefits to local communities be an effective way to promote biodiversity conservation. While the evidence is mixed, there are enough success stories to suggest that under a stable institutional framework, bioprospecting efforts in which local communities are fully involved can be an effective tool in order to help preserve certain biodiversity rich areas.

Along with climate change, biodiversity loss is probably the most pressing environmental issue currently facing the planet. Broadly defined, "*biodiversity encompasses the diversity of life forms present on the planet*". Traditionally, this has meant species diversity, but the definition can be broadened to include genetic diversity. The importance and visibility of biodiversity conservation as a crucial international issue have been greatly increased since the signing of the Convention on Biological Diversity (CBD) in Rio de Janeiro in 1992, and the CBD's definition best broaches the different views of biodiversity: '*biological diversity means the variability among living organisms from all sources, including interalia terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity between species, within species, and of ecosystems'*. The CBD also highlighted the fact that biodiversity protection and economic development issues are inexorably linked. This is due to the fact that the most important areas for biodiversity conservation fall almost entirely within the developing world. A survey of **Mittermeier *et al* 1997** has identified seventeen countries – which have been named "megadiversity" countries, which alone account for over 80% of the planet's biodiversity. The majority of these seventeen countries are in the developing world, particularly in the Andean region (Venezuela, Colombia, Ecuador, Peru), the Amazon basin (the above plus Brazil), and in south Asia (Malaysia, Philippines, Indonesia, India, China), along with several other large countries, most of which are part of the developing world.

While overall levels of biodiversity are obviously an important indicator of conservation importance, there are two other factors which need to be taken into account when identifying priority areas for biodiversity conservation. The first is levels of endemism, meaning the number of species which are found in a particular area and nowhere else in the world. The second is the level of threat faced by an area, as urgent conservation efforts need to be concentrated where the threats are most imminent. Particularly high levels of endemism are to be found in island ecosystems in countries such as Madagascar, Indonesia, Papua New Guinea and the Philipines, but key areas also include the Chocò ecoregion of Colombia and Ecuador, the Tumbesian region in Ecuador and Peru, the Upper Guinea rainforests of West Africa and many others.

Among the most highly threatened ecosystems are the Atlantic coastal forests of Brazil and, again, island ecosystems in countries such as the Philippines. It is easy to see how any discussion of global biodiversity conservation policies must take into account international development issues, and how conflict can easily arise between developed and developing countries when dealing with such issues. There are different types of efforts and strategies currently underway to preserve global biodiversity, and a brief overview, particularly with regards to how they can be linked to biotechnology development, is useful. The most widespread and the most effective way to preserve biodiversity is through, direct, *in situ* conservation that is, establishing protected areas where biodiversity levels are particularly high or threatened, and these are the types of conservation efforts recommended under the CBD.

However, this is not always as straightforward as it sounds, as the creation of protected areas inevitably means that access to natural resources is restricted. In order to better involve local communities in biodiversity conservation efforts, a number of market based conservation strategies have been developed, which aim to make biodiversity conservation profitable. The better studied ways to achieve this has been ecotourism, the attempt to promote sustainable, low impact tourism to protected areas as a a way of generating income for local communities that would then find economic incentives for the conservation of natural ecosystems. Bioprospecting, the exploration of poorly-known ecosystems aimed at finding potentially useful genetic material that can be used in biotechnology falls into such market based biodiversity conservation efforts. Furthermore, biotechnology can play an important role in *ex situ* conservation efforts, as one of the cornerstones of such

efforts is the establishment of gene banks which are vital to the success of the biotechnology industry.

Policy and Priorities of indigenous knowledge and benefit-sharing

Inadequacies among policies include blanket adoption of policies on these regions without considering the present culture, customs, practices and traditions. Advocacy is absent and seems to be adopted only as a political tool for delaying actual development. A few advocacy projects in the region are taken up by small NGOs and do not seem to have an impact on a regional scale. Various institutions dealing with utilization of resources need to be brought on a common platform to frame guidelines for sustainable use, IPR and benefit sharing and biotechnology policy.

Linkages need to be made between research institutions and key regulatory agencies for the region as a whole. Currently these linkages are somewhat blurred. Areas that should be addressed as priorities are biotechnology and benefit-sharing. Policies need to emerge from research outputs. Similarly linkages need to be established between various agencies carrying out development activities and regulatory agencies. Environmental impact assessment plans also need to be drafted specially for the Himalaya. An important systemic need for the Himalaya would be a region specific action plan. Inter-institutional linkages need to be improved for specific issues such as climate change. Studies on climate change require multidisciplinary inputs ranging from bio-physical sciences to socio-economic studies. Inter-institutional collaborations can also contribute to sharing scientific infrastructure and expertise, infrastructural development such as field stations and effective interdisciplinary research.

For the Himalayan region in particular specific policies are needed to address equitable benefit sharing, documentation and preservation of traditional knowledge (e.g., health, agro-pastoral, water conservation systems) and intellectual property rights. It is desirable that a separate set of policies be developed for the Himalaya. As a part of such a project, checklists, databases and status reports of species with commercial importance (especially medicinal plants) can be compiled. Policy formulation needs to be comprehensive and should be developed in conjunction with research institutions as well as all concerned higher level governmental departments to avoid contradictory policies. Efforts need to be made to communicate policy guidelines to the relevant customs departments and regulatory bodies.

In the absence of a comprehensive agro-biodiversity policy, the erosion of agro- biodiversity in the hilly regions of Uttaranchal is continuing unabated. Consequently, there is significant ecological degradation and furthermore, food security of poor farmers is threatened. The objectives of the project are: (i) exploration of the variety of agro ecosystem practices, (ii) the development of appropriate policy instruments that will promote the conservation of agro-biodiversity and achieve food security in the region. A central feature of the degradation of multiple ecological functions is a loss of natural and crop biodiversity in this fragile Himalayan ecosystem. The area under traditional crops has been declining and these have been replaced by cash crops. However, the popular notion is that access to roads in the hills reduces agro-biodiversity. The thrust of government policy instruments, like credit, subsidy, and the public distribution system, has been directed towards promoting high productivity monocultures.

India has a rich and varied heritage of biodiversity covering ten biogeographically zones, the trans-Himalayan, the Himalayan, the Indian desert, the semi-arid zone(s), the Western Ghats, the Deccan Peninsula, the Gangetic Plain, North-East India, and the islands and coasts (**Rodgers; Panwar and Mathur, 2000).** The COP to the Convention on Biological Diversity adopted a supplementary agreement to the Convention known as the Cartagena Protocol on Biosafety on 29 January 2000. The protocol seeks to protect biological diversity from the potential risks posed by living modified organisms (LMOs) resulting from modern biotechnology. It establishes an advanced informed agreement procedure for ensuring that countries are provided with the information necessary to make decisions before agreeing to the import of such organisms into their territory.

Environment and Biodiversity Conservation

In recent years, efforts of conservation are being made in the country. Many international organizations like IUCN, WWF, ICIMOD and KMTNC rendered help in this effort. By establishing national parks, wild life reserves, and botanic gardens measures of *in situ* conservation have been taken to protect plants animals *from* human encroachment. Such activities are very expensive and thus remained limited. Field gene banks are also costs a lot to maintain and moreover plants and animals maintained in such gene banks are susceptible to natural calamities. Diseases, cattle, herds, other animals, human encroachment and natural disasters often damage them. Therefore, conservation of rare and endangered species through multiple means is desirable. Biotechnological methods can be implemented to support *ex situ* and *in situ* conservation. The accessible means of biotechnological method in developing countries can be implemented in conservation of plant species. Application of hormones can promote propagation of rare species through seeds and other vegetative parts. Dissemination of propagated plants in wild is expected to make the measure of conservation a success. It is possible to maintain biodiversity *ex situ* using tissue culture, protoplast fusion, embryo transfer, cryopreservation and gene banks. The approaches of reintroduction of tissue culture raised plants and

establishment of gene banks in nature may thus be effective both *in situ* and *ex situ* conservation of plant genetic resources.

To facilitate absorption and utilisation of technology, major emphasis has been given on involvement of user industry and demonstration of technologies developed at the site of the industry. In over a dozen projects, a number of industries are involved in process development, process optimisation and validation. A number of technology packages such as ecorestoration of mine spoil dumps, microbial remediation of petroleum sludge and oil spill, phytoremediation of dye industry effluent treatment and palm oil mill effluent treatment have been standardised and are being negotiated for technology transfer.

Characterization and conservation of endangered species including medicinal and aromatic plants

A number of valuable plants species bearing food, fodder fuel wood, fiber and medicine are being used in huge amount by people. Adequate measures have not been taken yet to multiply and domesticate these plant species in order to conserve them. Genetic diversity of important species of the Himalayan region has been studied using molecular markers for conservation of these identified elite's in the Alpine region, field stations have been established in Himachal Pradesh – Rahala (2250 m) and Uttaranchal, Katochira, Distt. Almora (1850 m) and Khaljum, Dist Bageshwar (2450 m). These field stations are concentrating on the maintenance of germplasm and on farm cultivation of the elite material. Based on the genetic profiling studies, elites of *Aconitum heterophyllum, A. balfourii, Podophyllum hexandrum, Valeriana jatamansi, Gentiana kurroo and Picrorhiza kurroa* are now being taken up for mass multiplication at Rahala, H.P. and Almora. Morphological studies of *Podophyllum hexandrum*, *Valeriana jatamansi*, *Picrorhiza kuroa* and *Gentiana kurroo* have been done. The phenomenon of gynodioecism has been established in *Valeriana jatamansi*. In *Gentiana kurroo*, the peculiar mechanism of dichogamy has been established. Flowering in *Picrorhiza kurroa* occurs in two phases in May/June and August. Seed germination studies in *Podophyllum hexandrum*, *Valeriana jatamansi*, *Aconitum heterophyllum* and *Gentiana kurroo* have been done. The best sowing time for *P. hexandrum* and *A. heterophyllum* is November while as that for *V. jatamansi* and *G. Kurroo* is June. Besides, the use of conventional propagation methods, application of *in vitro* propagation techniques offers an additional alternative for recovery as well as multiplication of endangered species. Therefore, attempts were made to develop effective *in vitro* propagation protocols for *P. hexandrum*, *P. kurrooa* and *A. balfourii*.

The Network of the three gene banks set up by the DBT is fully equipped with state-of- the art facilities for conservation of seeds, live plants and *in vitro* material of rare, threatened and economically important species. A fourth gene bank has been established at RRL, Jammu to cover Western Himalayan Region. Under an integrated programme on taxol, about 1500 rooted stem cuttings of *Taxus* were planted in their natural habitats in Himachal Pradesh. The genetic diversity among *Taxus wallichiana* growing in the North Himalayan region has been estimated by RAPD analysis. This will help tagging high - yielding genotypes using DNA markers for micropropagation and mass multiplication. Various callus lines of *Taxus baccata* were screened by TLC/HPLC for taxol/10-DAB (a taxol precursor) production. Three promising cell lines were identified. The work carried out at NII; New Delhi has led to the identification and isolation of an isoquinoline alkaloid, berberine, an immunomodulatory agent from *Berberis aristata.* An MOU has been signed between NII and an industry for production of a herbal product. Permission from Drug Regulatory Authority is being sought for carrying out clinical trials in collaboration with the industry. A programme on "Biotechnological approaches for herbal product development" has been launched under the National Jai Vigyan Science & Technology Mission. It aims at developing improved ergot production technology, agrotechnologies for high yielding variety of *Artemisia annua* and developing herbal therapeutic products for curing hyperlipidemia and arthritis alongwith other immunomodulators.

Biological diversity in Himalayan region is closely linked to the livelihood and economic development of the people of hill areas and relates to agricultural productivity and sustainability. Countries with strong capacity in modern technologies would be interested for effective implementation of Plant variety Protection as envisioned in Trade Related Intellectual Property Rights (TRIPS) under the regime of World Trade Organization and, Private sectors investment is increasing in these countries because of increasing profit prospects in modern biotechnology sector where competition for exclusive rights for gene structures or gene sequences through patents is high. Countries rich in genetic resources, like India, would be more interested in implementation of the provisions of the CBD to honor the national sovereign rights on genetic resources, prior informed consent for the access of the material sharing of benefits and rewarding farming communities for their roles in conservation and management of genetic resources to meet their present needs and aspirations of future generations.

With the advent of substantial improvements in biotechnology and insufficient naturally occurring plants to meet the increasing demands of the medical markets, more wild medicinal plants with promising economic value have been identified and cultivated. Among these, wild yam (*Dioscorea* spp.) is a good example. The discovery of diosgenin, a steroidal sapogenin that occurs naturally in very high levels in

some yam species, led to a revolutionary means of synthesizing birth control agents. Since the strict Birth Control Plan was carried out in China from the 1970s onwards, demands for contraceptive pills increased very rapidly, leading to the investigation, analysis, cultivation, and processing of yams. Over-exploitation has threatened yams in the wild and they are now being cultivated in western Sichuan especially for diosgenin production. From 1996 until about 2000, the number of households in Maoxian County involved in the cultivation of wild yams rose to around 1,000.

Due to the simple skills required, a minimal input of labour, a guaranteed output of products, the fact that farming field space did not have to be taken up, more and more farmers are involved in this industry on a voluntary basis. Various schemes has contributed to farmers' participation in development projects sponsored by government or development agencies aiming at poverty alleviation in this region. The cultivation of high-value wild or introduced plants by farmers has played an important role in their economy. Meanwhile, the policy of encouraging diversified economic activities, as adopted by the provincial government in 1980, has also had a positive impact on the development of sideline production. When the state monopoly for the purchasing and marketing of all specialized local products (except musk) was rescinded in 1985, the farmers perceived this as a crucial incentive to exploit wild plant resources. Subsequently, business organizations at all levels have been engaged in the purchasing and marketing of all medicinal plants. Indigenous agro-ecosystems have played an important role in the conservation of biodiversity, and some ethnobotanical practices of agroforestry management have been integrated into the reforestation projects.

Cryopreservation

Experiments were initiated on cryopreservation of *A. heterophyllum* and *P. hexandrum* seeds collected in the year. In *A. heterophyllum*, the initial moisture percentage was low (6%) and were therefore directly stored under liquid nitrogen. The seeds were retrieved after regular intervals for evaluation of viability, germination and cryoinjury. Seeds showed about 90-100% germination after 30 days storage. However, there was a higher ion leakage in the seeds stored for 30 days as compared to the seeds stored for 10 days only. The seeds of *Podophyllum* had much higher initial moisture content (50%) and were therefore initially desiccated to 10 and 5 moisture levels and then stored in liquid nitrogen. Further time interval studies and development of protocols for liquid nitrogen storage were undertaken.

NOVEL PRODUCTS FROM WESTERN HIMALAYAS

Rhizosphere exploration for PGPR (Plant growth promoting responses) was often associated with enhanced plant growth and crop productivity, particularly under conditions of poor availability of mineral nutrients and stressful milieu. Aimed at developing plant growth promoting formulations for economically important crops of Lahaul and Spiti, evaluation of carrier-based microbial inoculants was initiated in multi-location trials. The microbial formulation was based on a consortium of efficient and stress tolerant phosphate-solubilizing and nitrogen-fixing rhizobacteria, selected for high PGPR activity under controlled environment. The phylogenetic relationships were worked out for these phosphate-solubilizing PGPR. Stress-tolerant and efficient phosphate-solubilizing bacterial isolateswere also subjected to diversity analysis with PCR-RFLPof 16S rRNAgene, employing the four-base-cutting restriction enzymes *Alu I*, *Rsa I*, *Hae III* and *Taq I*. Five distinct restriction patterns, with 3 to 5 restricted fragments/ pattern, were obtained with the restriction enzymes

Why biotechnology and biodiversity conservation are closely linked

Various institutes play a significant role in the conservation of plant resources in this region. To maintain their precious germplasm, a large number of medicinal and other economically useful plant taxa are grown in the medicinal-plant section as well as in its high-altitude extension. Agro-techniques for several of these and other potential bioprospective taxa have been developed for their successful mass propagation. Emphasis is laid on growing *ex situ* collections of Rare, Endangered and Threatened (RET) taxa of the region **(Dar & Naqshi 2002).** By virtue of these projects, a large proportion of our precious plant germplasm, collected from far-off and difficult habitats, has been maintained *ex situ*. Various ongoing research projects pertain to the conservation of medicinal plants, being funded by the Ministry of Environment & Forests (MoEF), Govt. of India, Department of Biotechnology (DBT) Govt. of India, and the G. B. Pant Institute of Himalayan Environment and Development (GBPIHED), Almora, India.

Biotechnology and biodiversity are undoubtedly closely linked, especially if one uses a broad definition of biotechnology that includes pharmaceutical uses of natural compounds, and not just genetic engineering. Broadly biotechnology is perhaps best defined as '*any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop micro-organisms for specific uses*' **(US OTA 1991).** However, because the pharmaceutical, agricultural, environmental and genetics industries are those who are funding the bulk of bioprospecting efforts, they will receive most of the focus **(Ernst & Young 1995)**.

The biotechnology industry's boom is a relatively recent one. The rise of firms devoted exclusively to biotechnology research and development started in the 1970's and gained momentum in the 1980's and especially the 1990's

(Acharya 1999). While in strictly economic terms multinational 5 companies remain far more important that small companies devoted exclusively to biotechnology (multinationals accounted for US$ 87 billion in annual sales in 1995, compared to US$ 9 billion for smaller firms), their rapid growth accurately reflects the growing importance of biotechnology, and such small companies are often at the forefront of bioprospecting efforts, and therefore particularly significant in light of links to biodiversity conservation.

As of now, the biotechnology industry is overwhelmingly located in the developed world. The lack of resources devoted to scientific research and a weak institutional regime in which public research institutions such as universities are poorly linked with private sector companies has made it difficult for biotechnology firms to establish themselves. This is particularly true when one looks at more modern biotechnology techniques – those of more interest to the pharmaceutical industry, and those most likely to be the focus of bioprospecting efforts – although a number of countries in east Asia, as well as Brazil, Mexico, Cuba, India, and China, are currently funding research in such areas. In other parts of the developing world, such as Africa, biotechnology research efforts are even further behind, and indeed most African countries have no institutions in charge of coordinating biotechnology research on a national level. It would be therefore expected for the biotechnology industry to reflect the views of developed countries in international forums dealing with trade and environment issues.

In order for biotechnology to exist as an industry, it needs a reservoir of biological and genetic material from which to draw its resources: the planet's biodiversity is therefore of fundamental importance to the industry. At the most basic level, many patents deposited by biotechnology firms are simply natural compounds found in certain plants and animals which may have beneficial medical, agricultural or other uses. In the US, the anti-coagulant properties of the venom of two Asian and South American pit vipers (*Agkistrodon rhodostoma* and *Bothrops athrox*) have been patented, while in Europe the therapeutic applications of extracts from the Indian plant *Cammiphora mukul* have also been patented **(Acharya 1999).** Both of these cases deals with compounds found in nature that can be used with little further elaboration from man, showing that often the biotechnology industry is dealing directly with the discovery and use of particular taxa.

Can the use of biotechnology promote biodiversity conservation?

This has led to the idea that biotechnology can help promote biodiversity conservation. The basic premise is that biodiversity contains hidden assets of potentially huge value to humanity, such as useful medical compounds found in plants or animals, new or better food crops. The search for new commercial applications for plant and animal species therefore gives biodiversity a significant enough innovation option value that biotechnology companies would be willing to pay for its preservation, and as such conserving a patch of biodiversity rich rainforest, for example, becomes more financially viable than converting it to farmland. The opportunity costs of biodiversity conservation would then be offset by the potential gains. Furthermore, as the private sector recognizes the economic value of biodiversity landowners and local communities in biodiversity rich areas will recognize the value and the potential benefits of their natural resources and will find it profitable to work towards their conservation.

Bioprospecting plays a key role in this argument. Bioprospecting refers to research undertaken in high biodiversity areas in order to discover potentially useful properties in local plants and animals, which can then be developed by the biotechnology industry. In many ways it is a form of basic scientific exploration. In fact, when one considers that only about 1.75 million of an estimated 14 million species of plants and animals have been described and named (World Conservation Monitoring Centre 2000; although some estimates run as high as 100 million species),bioprospecting can contribute to global biodiversity conservation in the most basic way, by contributing to the global inventory of known species. The compounds most sought after by the biotechnology industry are usually found in plants, of which 270 000 of an estimated 320 000 species have been described **(WCMC 2000).** This figure however is deceptive, as only about 25% of the world's estimated plant species are currently held in botanical gardens, and thus easily accessible to scientists **(Acharya 1999).** Furthermore, only a small percentage of these have been fully studied in order to identify their chemical properties, and bioprospectors are therefore taking the first steps in studying poorly-known taxa and contributing to global knowledge about biodiversity. The general way these efforts have been undertaken has been for a country to allow access to its genetic resources, and for prospectors to then identify and collect potentially useful taxa, which are then evaluated for potential use by the biotechnology industry. The countries from which these genetic resources come from are then compensated by the companies involved in the research, either through royalties paid on the use of commercially viable compounds, or though a "prospecting" fee paid in advance.

The argument that bioprospecting and biodiversity conservation could be positively linked first surfaced in the 1980's and gained strength in the 1990's. These arguments initially focused on the potential undiscovered economic value of biodiversity, especially with regards to the pharmaceutical industry. Early studies tried to estimate biodiversity's value to the pharmaceutical industry by estimating the

probability of discovering a commercially valuable substance, and multiplying it by the value of the discovery **(Simpson et al 1996)**. While the results of these studies were extremely variable, most suggested that the untapped economic potential of biodiversity was quite significant, with estimates running as high as US$ 27.3 million per untested species in situ **(Principe 1989).** The link between bioprospecting and biodiversity conservation was further bolstered by some important discoveries. A much-publicized example is that of the rosy perwinkle *Catharanthus roseus*, a wildflower native to Madagascar used in traditional medicine there. The plant was found to contain alkaloids active against leukemia, and it is now used to treat cancer, Hodgkin's disease and is the best known-treatment for childhood leukemia (the synthetic compound used to treat childhood leukemia is only 20% as effective as the natural alkaloids found in the rosy perwinkle). This discovery was highly touted and served to put bioprospecting on the map as a possible strategy to promote biodiversity conservation.

The discovery of the anti-cancer properties of the bark from the Pacific yew tree *Taxus brevifolia* was also crucial in highlighting the value of biodiversity to the pharmaceutical industry. The discovery of taxol not only strengthened the argument that important discoveries were still to be made through bioprospecting, but more importantly from the pharmaceutical industry's point of view it proved that such discoveries could be immensely valuable from a financial standpoint, not only as a short-term return on the initial investment but also as a long-term source of income. It also provided a blueprint for other bioprospecting and biodiversity development agreements between the private sector and government institutions **(Day-Rubenstein and Frisvold 2001).** In particular, the attention of pharmaceutical companies was quickly drawn towards the developing world, where the potential for new discoveries was far greater due to their richer ecosystems, whose levels of biodiversity were both far higher and far less studied than they were in developed countries. This however created artificially high expectations in many developing countries about the potential economic benefits to be achieved thanks to bioprospecting, and meeting such expectations has become a major issue in assessing the success of these efforts.

Other studies have built upon and refined **Simpson *et al* (1996)'**s model, in some cases changing some of the basic assumptions, and the results have been somewhat more encouraging. **Rausser and Small (2000)** expand upon Simpson et al's (1996) work by identifying a key flaw in their argument. Simpson *et al* assumed that research on the potential value of species was random, all species being tested in the same way; as a result, the odds of finding a valuable compound for any given species are extremely low. **Rausser and Small (2000)** however assume that research on the potential pharmaceutical value of previously unstudied species concentrates on those that are likely to contain useful compounds, through models that identify the most promising research areas and leads. Indeed, bioprospecting efforts do not take place randomly across the world, but are concentrated in areas and ecosystems where potentially useful taxa are expected to occur, or on species that are closely related to those that have already proven to be commercially viable – for example, taxol is now being extracted from the Himalayan yew tree, a close relative of the Pacific yew from which the drug was first extracted. The odds of finding useful compounds are therefore significantly higher than those calculated by **Simpson *et al* (1996),** who assumed that bioprospectors operated without prior information to help them focus their efforts. It suggests that bioprospecting can be an efficient incentive mechanism for conserving biodiversity rich areas. Furthermore, Rausser and Small (2000) suggest that firms' willingness to pay for bioprospecting rights may be even higher in light of the competition for patenting new discoveries, as firms may be willing to pay a premium for exclusive access to promising areas.

FUTURE STRATEGIES

The main question is whether bioprospecting can generate enough socially sustainable profits to serve as a useful tool for biodiversity conservation. An optimistic answer is that it can, at least on a local level, if the conditions are right. Early estimates on the economic potential of bioprospecting were widely divergent. The most optimistic of these, which suggested that profits from bioprospecting could be high enough to serve as a significant force for biodiversity conservation, have proven to be overstated. However, a number of carefully structured biodiversity sharing agreements have indeed been profitable, both for the private sector and for the developing country institutions with which they have been signed and can serve as blueprints for future such efforts. Indeed, it should be noted that one of the most successful aspects of such agreements, perhaps even more important than the financial rewards, has been the investment in capacity building in developing countries, and the benefits in training for local biodiversity conservation technicians that have arisen **(Porzecanski *et al*, 1999).**

In order for bioprospecting to fully contribute to biodiversity conservation, local communities, the ultimate stewards of biodiversity, must be fully involved and receive enough benefits to offset conservation costs. The major challenge for successful bioprospecting efforts in the future will be to more fully integrate local communities in their efforts. This issue has been relatively overlooked so far, and apart from a few success stories where agreements were negotiated directly with local communities (as was the case with the Kani people in India), local communities have often been by-passed. Involvement of these

communities is a critical step in protection of biodiversity.

The institutional framework in which these issues evolve is also crucial. At the moment, there is a conflict between the CBD, which reflects developing country interests and serves as a more effective framework for biodiversity conservation, and the TRIPs regime. Because of TRIPs' enforcement mechanisms, it is *de facto* the most relevant of the two in terms of influencing national and international policies. However, the CBD does have great resonance, and recent developments, such as the Doha conference, suggest that in the future TRIPs may move closer to CBD positions, thus providing a clearer and more favorable institutional framework. The outlook for the future of bioprospecting and biodiversity conservation is difficult to predict, but the fact that the issue remains at the forefront of current debate, and that there are ongoing developments on both the institutional and economic front, suggests that much work remains to be done. There is a growing realization of the need for a clearer institutional framework, and for better involvement of local communities, but until concrete steps are taken in this direction, success stories will remain scattered. Under ideal conditions, bioprospecting can be an effective way to preserve biodiversity locally, and it can play an effective, albeit limited role, in overall efforts to conserve global biodiversity.

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Prediction of Herbicide Sorption kinetics using GCMS Quantitation.

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Abstract: Economically Viable adsorption technique based on sorbate removal by phosphoric acid (H_3PO_4) poultry droppings (PD) was proposed to improve the ecological system. Two ways activation schemes generated activated carbons was applied to herbicide uptake and evaluated with three (3) kinetic models. GC/MS quantitation experiment based on external standard method was performed to demonstrate up to 89.216% atrazine removal. The kinetic equilibrium study showed that a 300 minute sorbate – sorbent interaction gave 19.293 out of 25 gdm^3 adsorption (being a 77.172% adsorption). This is only 5.140, 1.992, 1.432 and 0.632% higher than the 60, 120, 180 and 240 minutes interaction with 72.032, 75.180, 75.740 and 76.540% atrazine removal respectively .The pseudo-second order kinetics was observed to be more suitable in predicting the adsorption rate by the sorbate wherein the initial adsorption rate, h was estimated as 0.3646 $g.mg^{-1}$ min^{-1}. A time dependent physisorption phenomenon was evidenced. The major results support the conclusion that the sorted agro-waste has the potential to serve as extractants adsorbents in remediation process. [Life Science Journal 2010;7(3):29-36]. (ISSN: 1097-8135).

Key words: Quantitation, Adsorption kinetics, Rate, Poultry droppings, GCMS

Introduction

Atrazines and organophosphorus pesticides are considered as priority pollutants since they are harmful to organism even μgL^{-1} levels. These pesticides or herbicides constitute a diverse group of chemical structures exhibiting a wide range of physiochemical properties (Agdi *et al.*, 2000). Atrazine (2-chloro-4-, amino-6-isopropylamino-s-triazine) and related substituted chlorotriazine compound,2-chloro-4,6-bis(ethylamino)-s-triazine) finds extensive use as herbicides (Shimabukoro,1967).They are widely used for the control of broadleaf and grassy weeds. Contrary to expectations, these compounds reduce the rate of CO_2 fixation in plants and act as inhibitors of hill reaction during photosynthesis. Unfortunately too, it is also widely detected in water supplies **(**Itodo *et al.*, 2009a**).**

Among the conventional techniques for removing dissolved sorbates (heavy metals,dyes,organics etc.) include electrodialysis, phytoextraction (Myroslav *et al.*,2006).Others include ultrafilteration, reverse osmosis, chemical precipitation, ion exchange, carbon adsorption, evaporation and membrane adsorption.Most of this methods are expensive and ineffective when applied to low strength wastes with heavy metal concentration less than $100mgL^{-1}$ (Wong *et al.*,2000).Non conventional methods, studied for sorbate uptake include the use of wood, fullers earth, fired clay, fly ash, biogas waste slurry, waste orange peels, chitin, silica etc (Maria and Virginia, 2009).

Adsorption is the adhesion of a chemical substance (adsorbate) onto the surface of a solid (adsorbent). The most widely used adsorbent is activated carbon (Itodo *et al.*, 2009a).

Activated carbon can be prepared either by physical or chemical means, using a variety of starting material such as coconut shells, shell hull palm tree, apricot stones, almond shells etc with the most popular being wood charcoal or coal (Yoshiyuki and Yukata,2003).

Agricultural by – product is currently a major economic and ecological issue, and the conversion of these Agro products to adsorbent, such as activated carbon represents a possible outlet . This measure, to some extent, agrees with the concept of zero emission" as proposed to be an idea of reducing environmental impact produced by discarded waste products and increase the effective and repeated utilization of resources (Yoshiyuki and Yukata, 2003). About 9100 million tons of domestic animal manure

was generated in Japan in 2001. The average number of cattle and poultry wastes has increased by 56% and 176% in the world since 1978. These Poultry manure such as wastes and litters, in the absence of suitable disposal methods may pose a threat to the public health and the environment because of potential contamination of air, and ground and surface water sources via running off from the manure sites, and odor releases (Isabel *et al*.,2005).

To access adequately the feasibility of activated carbon for normal removal of contaminant, and to design the most effective manner in which it can be used ,it will be necessary to qualitatively and quantitatively predict the expected adsorption performance, using adsorption isotherms.(Dinesh and Charles,2007). Knowledge of adsorption kinetics (i.e. the rate of solute uptake, which dictates the residence times of sorbed solute at the solid-liquid interface) is important in carbon adsorption process.

Equilibrium is a phenomenon when the rate of adsorption and the rate of desorption are equal (Cooney, 1999). This is also the case when the effluent exiting an adsorption column contains pollutants at greater concentrations than is allowed. With a column system the adsorbent is said to be "spent."The relationship between the amount of adsorbate adsorbed onto the adsorbent surface and the equilibrium concentration of the adsorbate in solvent at equilibrium at a constant temperature may be estimated by various adsorption isotherm models. The amount of Dye at equilibrium, q_e was calculated from the mass balance equation given in equation 1 by Hameed *et al*.,(2006).

$$q_e = (C_O - C_e)\, V/W \ldots. (1)$$

where C_o and C_e are the initial and final Dye concentrations (mg/L) respectively. V is the volume of dye solution and M is the mass of the acid catalyzed Poultry waste sorbent (g). while t is the equilibrium contact time, when $q_e = qt$, equation 1 will be expressed as equation 2 below:

$$q_t = (C_o - C_t)v/w \ldots.(2)$$

where $q_e = q_t$ and C_t is the concentration at time t. The percent dye removal (RE %) was calculated for each equilibration by the expression presented as equation 3

$$R\ E(\%) = (C_0 - C_e)/Co \times 100, \ldots. (3)$$

Where R (%) is the percent of dye adsorbed or removed. The % removal and adsorption capacities were used to optimize the activation condition.(Maryam *et al.,* 2008). The test were done at a constant temperature of 25 ± 2^0C.(Rozada *et al*.,2002).

Adsorption dynamic and mechanism (Batch kinetic studies): Kinetics of adsorption is one of the important characteristics defining the efficiency of adsorption .According to Demirbas *et al.*, (2004), the study of adsorption dynamics describes the solute uptake rate and evidently the rate control the resident time of adsorbate uptake at the solid-solution interface. The adsorption rate constant can be used to compare the performance of activated carbons (Demirbas *et al*.,2004).Several models have been used by a number of authors to ascertain the kinetics and mechanism of adsorption onto activated carbon surface.

Experimental results were fitted to pseudo- First and pseudo-second order kinetic model. The integrated and linearized pseudo-first order kinetic model expression was given by Lagergren, (1898) in Ho and Mckay,(1999) as equation 4

$$\text{Log}\ (q_e - q_t) = \log q_e - (k_1/2.303)\ t\text{----- (4)}$$

On the other hand, Ho and McKay, (1999) presented the second-order kinetic model, integrated and linearized as 5

$$t/q = 1/k_2 q_e^2 + (1/q_e)\ t \ \text{----- (5)}$$

Where k_1 and k_2 stands for the pseudo first and second order rate constants respectively and were determined by regression analysis by fitting on a number of experimental data point, plotting log $(q_e - q_t)$ versus t and t/q against t From equation 4 and 5 respectively. q_e was calculated for each model and compared with its experimental value for precision validity test (Reuben and Miebaka, 2008 ; *Itodo et al*., 2009b,)

Validity tests: Accepted kinetic model for a given adsorption is characterized by three common validity test;

(i) A good and high correlation coefficient, R^2 indicating the applicability and reliability of a given model.
(ii) A close agreement between the calculated and experimental q_e values.
(iii) The accepted model must have the least values for the sum of error squares (% SSE), which is determined as equation 6

$$SS\ E\ (\%) = \sqrt{\sum (q_e.\text{exp.} - q_e\,\text{cal.})^2/N} \text{ ---- (6)}$$

Where N is the number of data points. (Hameed *et al*., 2006; Itodo *et al*;2009b).

Standardization for GC/MS: Quantitative analysis in gas chromatography is to convert the size of the peak into some measure of quantity of the particular material of interest. This involves chromatographing known amount of the material to be analyzed and measuring their peak sizes. Then, the composition of the unknown is determined by relating the unknown peaks to the known amounts through peak size. Standards are made from a matrix to be close to the unknown sample as possible not only in the amount of material to be analyzed, but also in the matrix of the sample itself. This standard was prepared, used and discarded within a short period of time owing to evaporation of most of the solvent and stability of standard (Robert and Eugene,2004).

External standardization for GC/MS: Techniques of external standardization entails the preparation of standards at the same levels of concentration as the unknown in the same matrix with the known. These standards are then run chromatographically under ideal conditions as the sample. A direct relationship between the peak size and

composition of the target component is established and the unknown was extrapolated graphically. This technique allows the analysis of only one component in the same sample. Peak size is plotted against absolute amount of each component or its concentration in the matrix (Robert and Eugene, 2004).

Materials and methods

Brand name herbicide (atrazine® presumably 2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-trazine) with assay of 50% atrazine was procured from a retailer's stand of the Agro-chemical wing of Sokoto central market, Nigeria. Stock standard solution (25g/L) was prepared and from which ranges of working standard were prepared in chloroform and stored in the dark. This was employed as adsorbate, used in this analysis. Zinc Chloride (98+ %) and Ortho Phosphoric acid obtained from prolabo chemicals were used as chemical activants while Chloroform was used as solvent. Hydrochloric acid (0.1M) and distilled water were used as washing agents.

Sample collection and preparation of activated carbon: Poultry droppings, PD (as the raw material for the production of activated carbon) were collected from Labana farms, Aliero in Kebbi state. The raw materials were pretreated as earlier described elsewhere (Zahangir *et al*.,2008;Itodo *et al* .,2009a and b). For thermo chemical (heat/chemical) activation, methods by Itodo *et al*., 200a and 2009b;Turoti *et al*.,2007 were used after slight modifications. The samples (activated carbon produced) were crushed and sieved using <2mm aperture size sieve.

Preparation of Atrazine standard: 5 g of substrate was diluted to the mark of $100cm^3$ volumetric flask. This concentration of 50g/L herbicide is equivalent to 25g/L or 25,000ppm atrazine stock.

Batch equilibrium kinetic studies: Accurately 0.1g of home based activated carbon was mixed with $10cm^3$ of the 25g/L atrazine solution. The residual concentration of atrazine in solution (C_e in g/L) was measured after different stirring and interaction times (60, 120, 180, 240, and 300mins). The equilibrium phase herbicide was analyzed using a GC/MS. External standard method was used to calibrate the machine beforehand (Min and Yun,2008;Agdi *et al*.,2000).

GC/MS Conditioning: A gas chromatography equipped with a mass spectrophotometer detector (with a model GCMS QP2010 plus Shimadzu, Japan) was used in this analysis. The column was held at 60^oC in injection volume of 1µL and then programmed to 250^oC. it was set at a start m/z of 40 and end m/z of 420.The detector (mass spectrophotometer) was held at 250^oC above the maximum column temperature. The sample size was 1µL, which was split 100^{-1} onto the column and so the total charge on the column was about 1. Helium was used as the carrier gas at a linear velocity of 46.3cm/sec and pressure of 100.2kPa. Ionization mode is electron ionization (EI) at a voltage of 70eV. In this analysis, Amplification and resolution for test herbicide was achieved by adjusting the threshold to 6000. Thus, worse interference and solvent peaks were screened out leaving majorly the deflection of target compound (atrazine) as it was made pronounced on the chromatogram. Baseline disturbance was linked to either hydrocarbon impurities. Impure carrier gas can also cause baseline instability (Robert and Eugene, 2004).It can be corrected by changing the purifier when pressure drops reaches 10 – 15 pSi routinely monitoring the pressure. Sorption efficiency of an adsorption process was defined based on the fractions of extracted and unextracted sorbates (Robert and Eugene, 2004).

Calibration curve for GC/MS analysis: A three point calibration curve was made from 1.0, 5.0 and 10.0g/L atrazine solution. These standards were run chromatographically under ideal conditions. A direct relationship between the peak height or size and concentration of target was established. The unknown was extrapolated graphically (Robert and Eugene, 2004).

This work was aimed at evaluating Poultry Droppings as a substrate for removing atrazine (herbicide) from aqueous solutions or water environment. The specific objectives include; Generation of activated carbon thereby adding values to the wastes. Testing the experimental data with3 different kinetic models viz; (i) First order kinetics, (ii) Second order kinetics and (iii) Apparent first order kinetics. Beside adding value to the waste and arriving at a more ecofriendly environment, contribution by this work was also hoped for its scholarly knowledge in areas like prediction of kinetic models, transport models, sorption energies and their evaluations.

Results and Discussion

Experimental results were fitted to pseudo- First and pseudo-second order kinetic model. The integrated and linearized pseudo-first order kinetic model expression were given earlier by Lagergren, (1898) as equation 4.

The results from Figure 2 showed that the equilibrium phase concentration at any time C_t. the mass of the unadsorbed sorbates reduces (from 0.0693 – 0.0571) as contact time increases from 60 – 300 minutes. This implies that the removal efficiency increases (from 72.032% - 77.1325%) with time. Table I shows the effects of sorbate – sorbent contact time on the sorption efficiency.

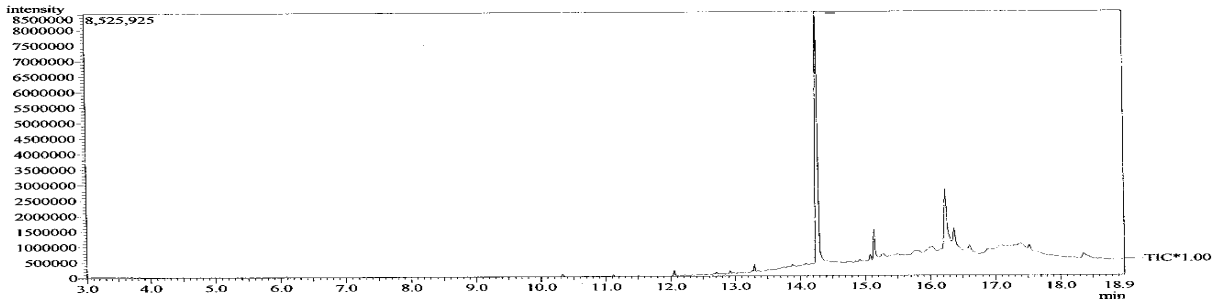

Figure 1: GC/MS chromatogram of equilibrium concentration atrazine after adsorption onto PD/A/60min. sorbent (*Carrier gas-Helium 100.2kpa,Column temperature –60°c,Injection temperature-250°c,Injection volume—1µL,Flow rate-1.61mL/min, Injection method- split, Linear velocity- 43.6cm/sec.*)

Effects of contact time on sorption efficiency (%RE)

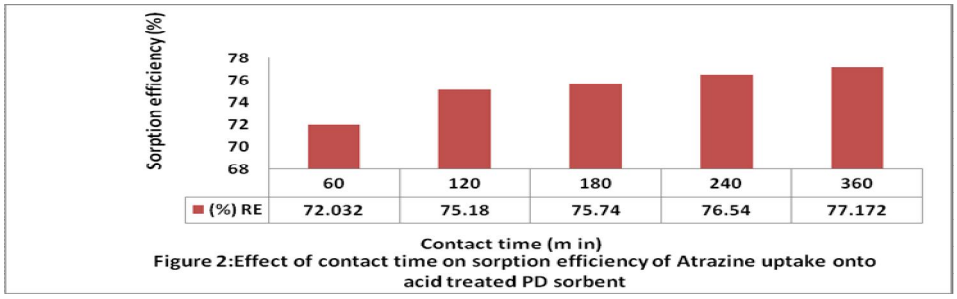

Figure 2:Effect of contact time on sorption efficiency of Atrazine uptake onto acid treated PD sorbent

Table 1: Adsorption experimental data of atrazine uptake by fixed mass of PD-Sorbents at different contact time, using GC/MS.

Biosorbent	Co(g/dm^3)	Ct (g/dm^3)	Ca (g/dm^3)	% RE	Ads mass (mgx10^{-3})	q_t (mg/g)	Kc= Ca/Ct	F = q_t/q_e
PD/A/60	25	6.925	18.008	72.032	0.1801	1.801	2.600	0.9385
PD/A/120	25	6.205	10.795	75.180	0.1879	1.879	3.029	0.9740
PD/A/180	25	6.065	18.935	75.740	0.1894	1.894	3.122	0.9819
PD/A/240	25	5.865	19.135	76.540	0.1914	1.914	3.263	0.9922
PD/A/300	25	5.707	19.293	77.172	0.1929	1.929	3.381	1.000

PD/A/60 – Poultry droppings, treated with, H_3PO_4interacted with Atrazine solution for 60 minute. PD/A/300 – Poultry droppings, treated with, H_3PO_4interacted with Atrazine solution for 300 minute.

Chromatograms presented as Figures 3 to 7 were typical of charts obtained for the equilibrium phase concentration analyzed using GC/MS. Analysis was carried out after filtration at the 60,120,180,240 and 300th minutes contact time. As interaction time increases, equilibrium concentration reduces. This implies an increase in adsorbed sorbate concentration with time.

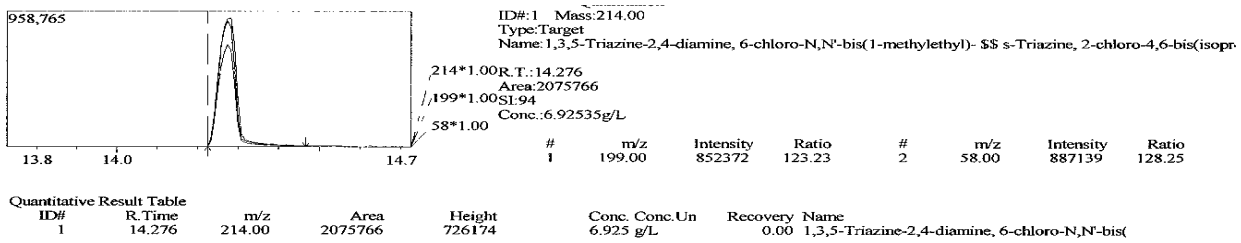

Figure 3: GC/MS chromatogram, quantitative measurement and spectral information of equilibrium phase atrazine after adsorption onto PD/A/60min sorbent

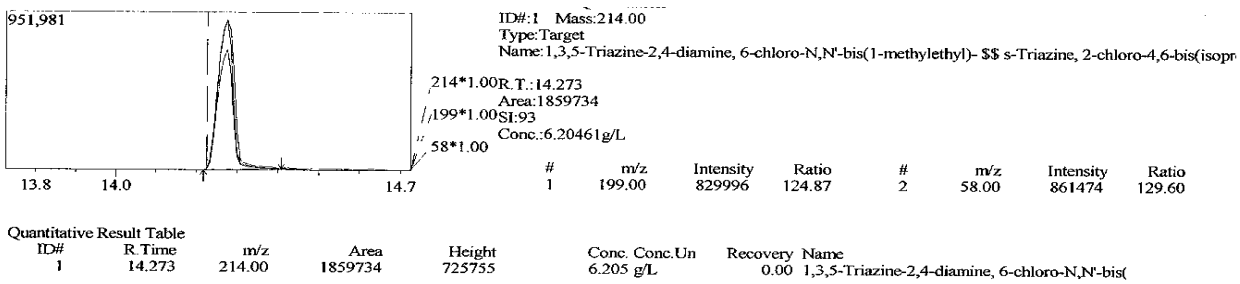

Figure 4: GC/MS chromatogram, quantitative measurement and spectral information of equilibrium phase atrazine after adsorption onto PD/A/120min sorbent

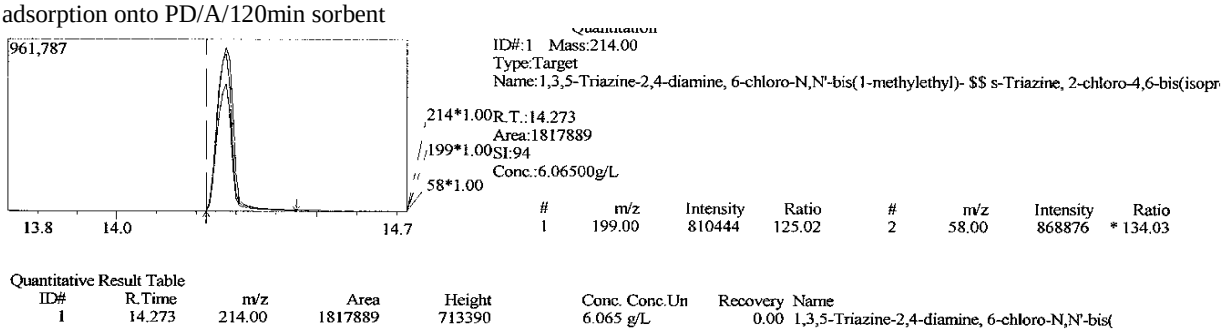

Figure 5: GC/MS chromatogram, quantitative measurement and spectral information of equilibrium phase atrazine after adsorption onto PD/A/180min sorbent

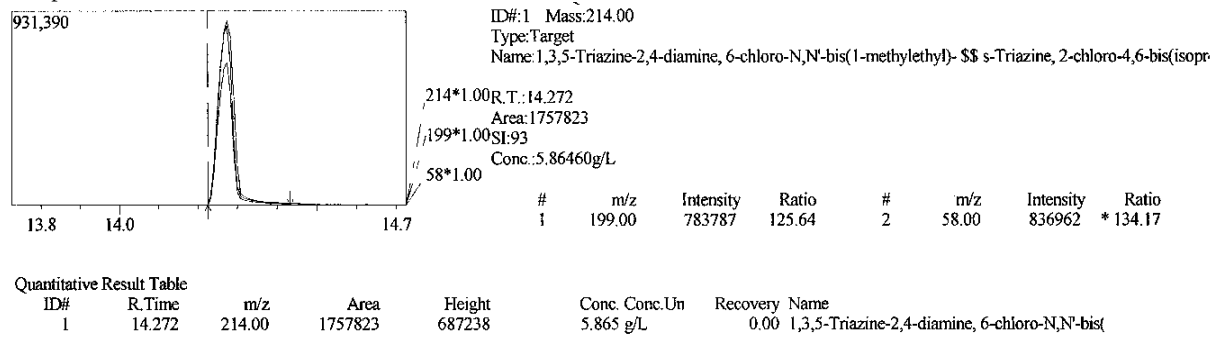

Figure 6: GC/MS chromatogram, quantitative measurement and spectral information of equilibrium phase atrazine after adsorption onto PD/A/240min sorbent

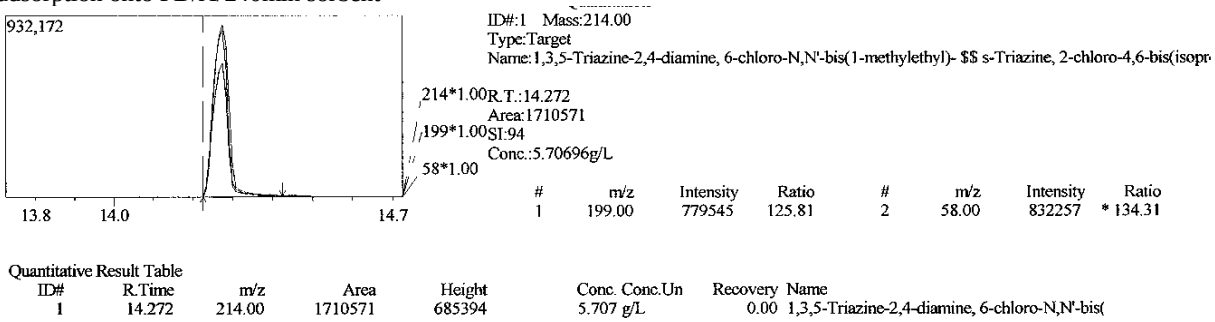

Figure 7: GC/MS chromatogram, quantitative measurement and spectral information of equilibrium phase atrazine after adsorption onto PD/A/300min sorbent

The extent at which contact time affect adsorption is non monotonical in multiplicity. At such, a 300 min sorbate – sorbent interaction gave 19.293 adsorption (being a 77.172% adsorption). This is only 5.140, 1.992, 1.432 and 0.632% higher than the 60, 120, 180 and 240 mins interaction with 72.032, 75.180, 75.740 and 76.540% atrazine removal respectively. Summarily, over 72% of atrazine removal was attained within the selected 60 – 300mins timing.

Effect of contact time on rate studies: Result clearly indicates that sorption by PD/A sorbents presents good uptake > 70% along the series (60-300 minutes contact time). However, Table 1 gave results which revealed that the sorption efficiency by PD/A/300 (77.00%) > PD/A/240 (96.540%) … while the least was by PD/A/60 (72.032%).

The initial adsorption rate, h was estimated from the second order kinetic model as 0.3646 $g.mg^{-1}$ min^{-1} while the fractional attainment at equilibrium was calculated as $F = q_t/q_e$ (Juliade *et al.*, 2008). F is a measure of the diffusion coefficient, D (cm^2/s). In this analysis, the F (Table 2) values tend to unity as the interaction time increases. Equilibration is attained at 300 minutes with F value of 1 while 60, 120, 180 and 240 contact time gave value of 0.934, 0.974, 0.982 and 0.992 respectively.

Table 2: kinetic experimental data of atrazine uptake onto PD/A-sorbent by fixed mass Sorbents at different contact time, using GC/MS.

t(min)	lnt	$t^{1/2}$	Ct	q_t x10^{-3}	$1/q_t$ x10^{-3}	t/q_t x10^{-3}	log(q_e - q_t)	F= q_t/q_e	ln (Ca/Ct)
60	4.094	7.746	6.925	1.801	0.555	33.315	-0.893	0.934	0.9755
120	4.787	10.954	6.205	1.879	0.532	63.864	-1.301	0.974	1.108
180	5.193	13.416	6.065	1.894	0.528	95.037	-1.456	0.982	1.138
240	5.481	15.492	5.865	1.914	0.522	125.392	-1.824	0.992	1.183
300	5.704	17.321	5.707	1.929	0.518	155.521	-	1.000	1.218

Where F = fractional attainment at equilibrium. Adsorption tends to equilibrium as F values approaches unity (1.0). F can be used to measure effective diffusibility.

This behavioral trend is corroborated by the findings of Ho and McKay, (1999) that sorption is influenced by contact time, pH, sorbent concentration, nature of solute and its concentration as re-emphasized by Reuben and Miebaka, 2008 ; Itodo *et al*., 2009d). In this study, it was evidence that equilibrium time for the chosen concentration (25g/L) and sorbent dose (0.1g) in $10cm^3$ aliquot is 300 minutes, wherein 19.293g/L out of 25g/L atrazine was adsorbed. Cossich, (2002) reported equilibrium time to range between 15 minutes and 10 days.

Table 3: First order, Second order and Apparent first order kinetics experimental constants of atrazine uptake onto PD/A-sorbent by fixed mass of Sorbents at different contact time, using GC/MS

Kinetic model	Relationship (y =)	R^2	Constants	Values
First order	-0.004x – 0.630	0.974	$k_1(min^{-1})$	0.00921
			$q_e(cal)(mgg_{-1})$	0.234
			$q_e(exp)(mgg^{-1})$	1.929
			%SSE	0.758
Second order	0.510x – 2.743	1.00	$k_2(mgg^{-1}min^{-1})$	0.0948
			$h(g\ mg^{-1}min^{-1})$	0.3646
			$q_e(cal)(mgg^{-1})$	1.9607
			$q_e(exp)(mgg^{-1})$	1.929
			%SSE	0.014
Apparent first order	0.001x + 0.940	0.876	$Ka(min^{-1})$	0.001
			C	0.940

In line with the aforementioned validity tests, It was observed that the value of the equilibrium concentration deduced with the pseudo-first-order kinetics is q_e = 1.929 mg/g while its experimental value is 0.234 mg/g (low precision).Estimated value of the correlation coefficient (R^2 = 0.974).This is also comparably low while the statistical sum of error is higher (%SSE=0.758) for the first order kinetics. On the other hand, calculated q_e value for the pseudo-second-order kinetic (q_e = 1.920) is of high precision to the experimental q_e (1.9607) units in mg/g. A perfect correlation of 1.00 and extremely low statistical sum of error was evaluated and reported (Table 3).This results shows that the pseudo-second order kinetic model is more suitable in predicting atrazine sorption from aqueous medium. Similar result was reported by Hameed *et al.,*(2006).

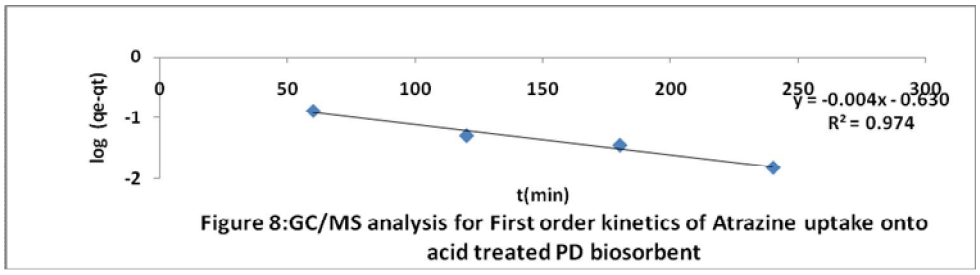

Figure 8:GC/MS analysis for First order kinetics of Atrazine uptake onto acid treated PD biosorbent

Generated data were also fed into the apparent first order equation, as described (Ana *et al*., 2009) in equation 7.

$\ln (C_o/C_e) = K_a t$ --------(7)

The wide range of apparent first order rate constant (0.001) as compared to that of the pseudo first order kinetics (0.00921) is also an indication that the sorption of atrazine onto PD sorbent does not follow the first order kinetics.

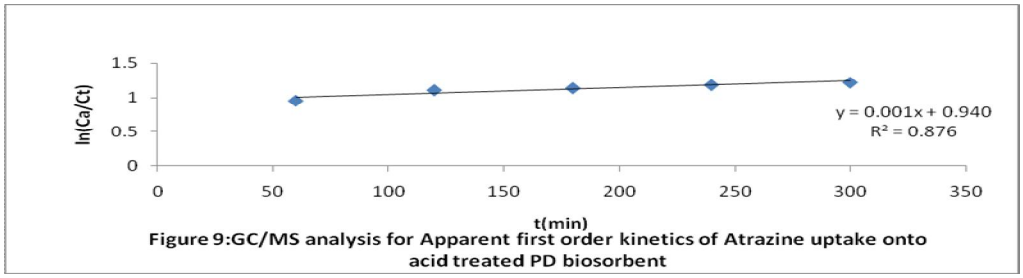

Figure 9:GC/MS analysis for Apparent first order kinetics of Atrazine uptake onto acid treated PD biosorbent

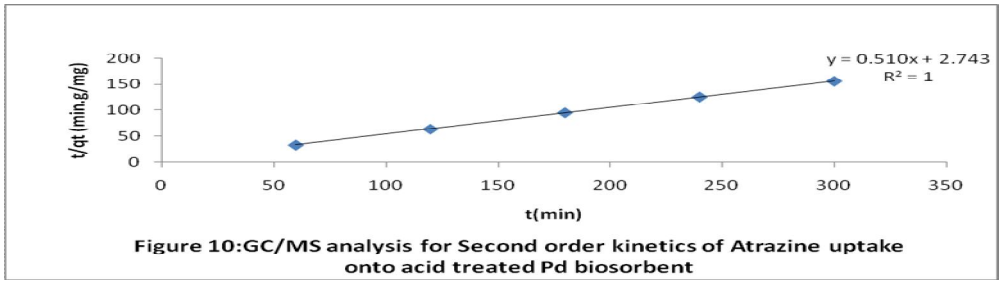

Figure 10:GC/MS analysis for Second order kinetics of Atrazine uptake onto acid treated Pd biosorbent

Conclusion: The PD activated carbon matrices generated, using the two ways activation scheme with acid (H_3PO_4) and salt ($Zncl_2$) as activating agents was successfully prepared and reported to provide over 70% atrazine adsorption for a kinetic study of 60min-300min. Contact time. A second order kinetic process was reported. An external standard preparation, followed by GC/MS quantitation proves a good option for analyzing the quantity of single analyte from a multicomponent system such as atrazine from either herbicide or pesticide.

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Chlorpyrifos-Induced Clinical, Hematological and Biochemical Changes in Swiss Albino Mice- Mitigating effect by co-administration of vitamins C and E

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Short title: Vitamins C and E mitigate chlorpyrifos-induced pathological changes

Abstract

Background. Induction of Oxidative stress is one of the molecular mechanisms in chlorpyrifos toxicity. *Objective*. To evaluate the effect of prolonged CPF exposure on clinical, hematological and biochemical parameters in mice and the possible ameliorative effect of coadministration of vitamins C and E. *Methods*. 40 mice divided into 4 groups of 10 animals in each group served as subjects for this study. Groups I and II were administered corn oil (2 ml/kg) and combination of vitamins C (100 mg/kg) and E (75 mg/kg), respectively. Group III were exposed to CPF only (21.6 mg/kg ~ 1/5th of the previously determined LD_{50} of 108 mg/kg), while group IV were pretreated with combination of vitamins C (100 mg/kg) and E (75 mg/kg) and then administered CPF (21.6 mg/kg) 30 min later. The regimens were administered orally once daily for a period of 10 weeks. The mice were examined for signs of toxicity and weekly body weight changes. Blood and serum samples obtained from sacrificed animals at the end of the study were evaluated for some hematological and biochemical parameters, respectively. *Results*.Vitamins pretreatment ameliorated cholinergic toxic signs and changes in body weight, PCV, Hb, RBC and WBC count induced by CPF. CPF-evoked alteration in Na^+, K^+, Cl^-, TP, urea, creatinine, ALP and MDA levels were ameliorated by pretreatment with the vitamins. ALT and AST activities lowered by CPF was further reduced by vitamins pretreatment. *Conclusion*. Vitamins C and E protected mice from subchronic CPF-induced alteration in clinical, hematological and serum biochemical parameters. [Life Science Journal 2010;7(3):37-44]. (ISSN: 1097-8135).

Keywords: Chlorpyrifos; hematology; serum biochemistry, lipid peroxidation; vitamins C and E.

Abbreviations
CPF= Chlorpyrifos
OP= organophosphate
MDA= Malonaldehyde
PCV= Packed cell volume
Hb- Hemoglobin
RBC= Red blood cells
WBC= White blood cells
Na^+= Sodium
K^+= Pottasium
Cl^-= Chloride
TP= Total proteins
AST= Aspartate aminotransferase
ALT= Alanime aminotransferase
ALP= Alkaline phosphatase

Introduction

Chlorpyrifos (CPF) (*O,O*-diethyl 0-[3,5,6-trichloro-2-pyridinol phosphorothionate) is a broad-spectrum OP insecticide that is widely used in agriculture and domestic pest control [1]. Toxicity associated with this insecticide led to the restriction of some of its domestic uses by United State Environmental Protection Agency in 2000. Despite its restriction, CPF still remains one of the most widely used insecticides. According to Steenland et al. [2], CPF is applied about 20 million times per year in US to houses and lawns, and 82% of adults have detectable levels of the 3,5,6-trichloropyridinol, the metabolite of CPF in their urine. Like the other OPs, CPF toxicity has been largely associated with irreversible inhibition of acetylcholinesterase (AChE) resulting in accumulation of acetylcholine in the cholinergic receptors[3]. However, other putative mechanisms have been implicated in molecular mechanisms of CPF toxicity. Among these, the induction of oxidative stress has received tremendous attention[4-8].

The mammalian cells reduced the adverse effect of lipid peroxidation via the utilization of both enzymatic and non-enzymatic antioxidants, which scavenge for free radicals in the system. Oxidative stress results when the endogenous antioxidants have been overwhelmed by the rate and extent of free radical generation. Therefore, during oxidative stress, an increase in the exogenous supply of antioxidants improves the capacity of the tissue to cope with high antioxidant demands. Several studies have suggested high effectiveness following administration of two antioxidants in combating oxidative stress in the body[9-10]. It has been shown that the combination of vitamins C and E reduced lipid peroxidation induced by CPF[4-6]. We have earlier demonstrated the ameliorative effect of vitamin C on some of clinical, hematological and biochemical changes induced by repeated CPF administration in mice[7]. Therefore, the aim of this study was to evaluate the effect of CPF on clinical, hematological and serum biochemical changes in mice, and the possible ameliorative effect of the combination of vitamins C and E.

Materials and Methods

Chemicals

Commercial grade CPF (Termicot®, Sabero organics, Gujarat Limited, India), Vitamin C tablet (Medvit C®, Dol-Med Laboratory, Nigeria) and vitamin E (α-tocopherol, Paterson Zochonis, Nigeria) were used for this study. Both

the CPF and vitamin E were reconstituted appropriately in corn oil immediately prior to use.

Animals and Treatments

Forty Swiss albino mice of both sexes weighing between 17 and 21g served as subjects for this study. The mice were fed on standard mice pellets and water was provided *ad libitum.* They were randomly divided into four groups. Group I (control) received corn oil only (2 ml/kg) while group II (VC+VE group) were co-administered vitamins C (100 mg/kg) and E (75 mg/kg). Group III (CPF group) received CPF only (21.6 mg/kg~ equivalent of 1/5th LD_{50} of 108 mg/kg determined in the preliminary study). Group IV (VC+VE+CPF group) were pretreated with coadministred vitamins C (100 mg/kg) and E (75 mg/kg) followed by exposure to CPF (21.3 mg/kg), 30 minutes later[7,11]. These regimens were administered *per os* three times every week days (Mondays, Wednesdays and Fridays) for a period of ten weeks. During the test period, the animals were observed for any abnormal clinical signs and death, and body weight changes evaluated on weekly basis. The experiment was performed according to the guidelines on animal research of the Animal Research Ethic Committee of the Ahmadu Bello University, Zaria.

Evaluation of hematological parameters

At the end of the test period, the mice were sacrificed by decapitation after light ether anesthesia, and blood samples (2 ml) collected into heparinised sample bottles were examined for packed cell volume (PCV), hemoglobin (Hb) concentration, total red blood cells (RBC) and absolute and differential white blood cell (WBC) counts using the method described by Dacie and Lewis[12].

Evaluation of serum biochemical parameters

Another set of blood samples collected into test tubes were allowed to clot and then centrifuged at 1000 x g for 10 minutes to obtain the serum. The serum was evaluated for the levels of TP, electrolytes (Na^+, K^+ and Cl^-), urea, creatinine, AST, ALT and ALP. AST and ALT were determined using the method of Reitman and Frankel[13], while ALP was evaluated according to the method of King and Armstrong[14]. Serum creatinine was measured as described by Miller and Miller[15], urea was determined using the modified method of Natelson [16], using diacetyl-monoxime-thiosemicarbazide procedure. In addition, the serum Na^+ and K^+ were measured by flame photometry, while Cl^- was analysed using the method of Schales and Schales [17].

Evaluation of serum malonaldehyde concentration

Serum malonaldehyde (MDA) concentration as an index of lipoperoxidative changes was evaluated using the method of Draper and Hadley[18] as modified[19]. For this purpose, 1.25 ml of 100 g/L trichloroacetic acid solution was added to 0.25 ml serum in each centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 x g for 10 min, and 1 ml of the supernatant was added to 0.5 ml of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance measured using a UV spectrophotometer (Jenway, 645, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5 /cm, and expressed in μmol/ml.

Statistical analysis

Values obtained were expressed as Mean ± SEM and then subjected to one way analysis of variance followed by Tukey's multiple comparison test. The mean body weight of the mice in each group at the commencement of the study (week I) was compared with that obtained at the termination of the study (week X) using the Student's *t*-test. The statistical analysis was done using graphpad prism version 4.0 (www.graphpad.com). Values of $P<0.05$ were considered significant.

Results

Effects of treatments on clinical signs

The control and VC+VE groups did not show any apparent sign of toxicity. Toxic signs observed in the CPF group included huddling, depression, conjunctivitis, mild tremor, piloerection, soft fecal bolus (mild diarrhea) and dyspnea. Death occurred in two of the mice at 7th and 9th weeks of dosing, respectively. VC+VE+CPF group showed milder toxic signs compared to those in the CPF group, and these included huddling, depression, rough hair-coat and tremor.

Effect of treatments on body weight changes

The effect of the treatments on body weight changes is shown in Figures 1 and 2. A consistently progressive increase in body weight was recorded in mice in the control, VC+VE and VC+VE+CPF groups. A significant increase ($P<0.01$) in body weight gains was recorded at termination compared to at commencement of the study in the control, VC+VE with percentage weight increase of 32% and 42%, respectively. The CPF group showed a less progressive increase in their dynamics of body weight gain over the ten week period, and there was no significant change in their body weight at termination (21.4 ± 2.2g) compared to the value obtained at the commencement (20.8 ± 3.5g) of the study, with a percentage weight increase of 3%. On the other hand, VC+VE+CPF group demonstrated a progressive elevation of body weight gain over the study period, and there was a significant increase ($P < 0.01$) in body weight at termination (24.5 ± 2.9g) compared to that obtained at the commencement (19.5 ± 3.5g) of the study with a percentage body weight increase of 20%.

Effect of treatments on hematological parameters

The effect of the various treatments on PCV, Hb and RBC concentrations is shown in Figures 3, 4 and 5, respectively. A significant increase in PCV ($P < 0.05$), Hb concentration ($P < 0.01$) and RBC counts ($P < 0.05$) was recorded in the CPF group compared to the control. The PCV, Hb and RBC concentrations in VC+VE+CPF group were not significantly different ($P> 0.05$) from those obtained in the control and VC+VE groups. There was a significant decrease in PCV ($P < 0.01$), Hb ($P < 0.05$) and RBC ($P < 0.01$) in the VC+VE+CPF group compared to the CPF group. The WBC in the CPF group was significantly lower ($P < 0.01$) than those obtained in the control, VC+VE and VC+VE+CPF groups, respectively. Differential leukocyte count showed that neutropenia was the cause of leukopenia observed in the CPF group. On the other hand, the WBC concentration in the VC+VE+CPF group was not significantly different ($P> 0.05$) from those obtained the control and VC+VE groups, respectively. Similarly, there was a significant elevation ($P < 0.01$) in WBC in the

VC+VE+CPF group compared to the CPF group (Figure 6).

Effect of treatments on serum biochemical parameters

A significant increase ($P < 0.01$) in the concentration of Na^+ was obtained in the control compared to the VC+VE and VC+VE+CPF groups, respectively. The Na^+ concentration in the CPF group was significantly higher compared to VC+VE and VC+VE+CPF groups, respectively. K^+ concentration in the control group was not significantly different from those obtained in the CPF and VC+VE+CPF groups. However, the K^+ concentration in the CPF group was significantly higher ($P < 0.01$) compared to VC+VE+CPF groups. There was no significant change in the Cl^- concentration in the CPF group compared to the control and VC+VE groups, respectively. However, a significant increase ($P < 0.05$) in the Cl^- concentration was obtained in the CPF group compared to the VC+VE+CPF group (Figure 7).

The TP concentration was significantly higher ($P < 0.05$) in the CPF group compared to the control and VC+VE groups, respectively. No significant change ($P > 0.05$) in the TP concentrations was obtained in the VC+VE+CPF group compared to the control (Figure 8).

The urea concentration in the VC+VE+CPF group was not significantly different ($P > 0.05$) from those obtained in the control and VC+VE groups. However, there was a significant increase ($P < 0.01$) in the urea concentration in the CPF group compared to the VC+VE+CPF group. The creatinine level in the CPF group was significantly increased ($P < 0.01$) compared to the control and VC +VE groups. Similarly, a significant rise ($P < 0.01$) in creatinine concentration was obtained in the VC+VE+CPF group compared to the control and VC+VE groups, respectively (Figure 9).

The effect of treatments on serum enzyme's activity is shown in Figure 10. There was a significant reduction ($P < 0.01$) in the activity of ALT in the CPF group compared to the control. The ALT activity was also significantly lowered ($P < 0.01$) in the VC+VE+CPF group compared to the control and CPF groups, respectively. The level of AST in the control group was significantly higher ($P < 0.01$) compared to the CPF and VC+VE+CPF groups, respectively. The activity of ALP in the CPF group was significantly elevated compared to the control ($P < 0.05$), VC+VE ($P < 0.01$) and VC+VE+CPF ($P < 0.01$) groups, respectively.

Effect of treatments on serum malonaldehyde concentration

The effect of treatments on serum thiobarbituric reactive acid substance, MDA is shown in Figure 11.The serum MDA concentration was significantly increased ($P < 0.01$) in the CPF group compared to the control, VC+VE and VC+VE+CPF groups, respectively. No significant change ($P > 0.05$) in the MDA concentration was recorded in the control group compared to the VC+VE and VC+VE+CPF groups, respectively.

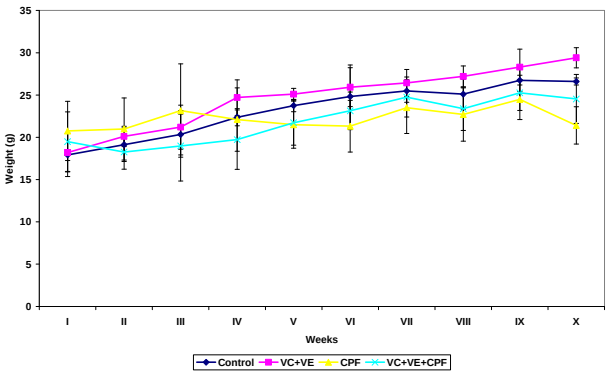

Figure 1: Effects of chlorpyrifos and the combination of vitamins C and E on dynamics of body weight throughout the period of study

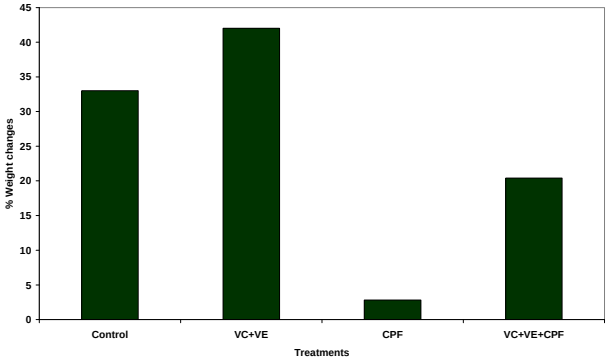

Figure 2: Percentage weight changes of mice administered chlorpyrifos (CPF) and vitamins C (VC) and E (VE)

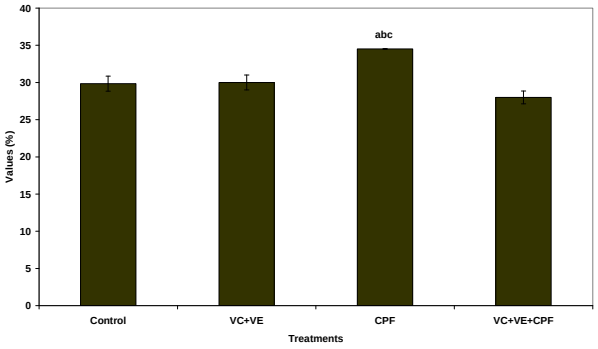

Figure 3: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on packed cell volume in mice. [a] $p < 0.05$ versus control; [b] $p < 0.05$ versus vitamin C+vitamin E group; [c] $p < 0.05$ versus vitamin C+vitamin E+CPF group

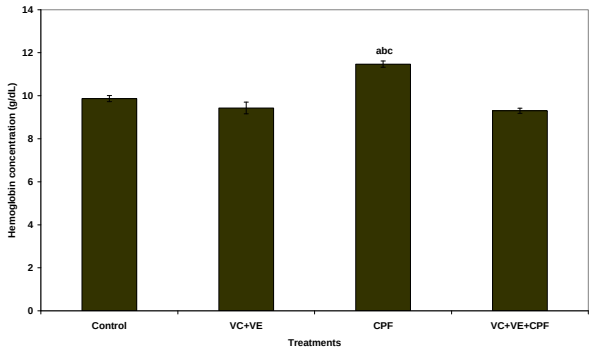

Figure 4: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on hemoglobin concentration in mice. [a] $p < 0.05$ versus control; [b] $p < 0.01$ versus vitamin C+vitamin E group; [c] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group.

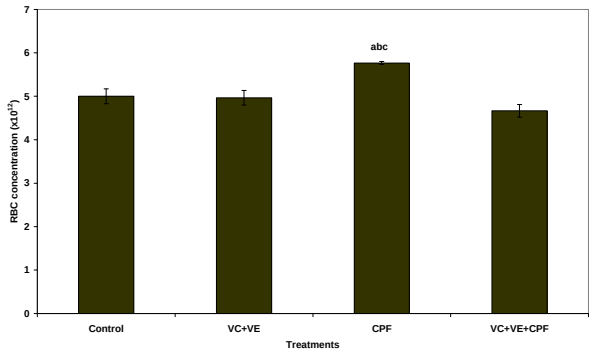

Figure 5: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on red blood cell count in mice. [a] $p < 0.05$ versus control; [b] $p < 0.05$ versus vitamin C+vitamin E group; [c] $p < 0.05$ versus vitamin C+vitamin E+chlorpyrifos group.

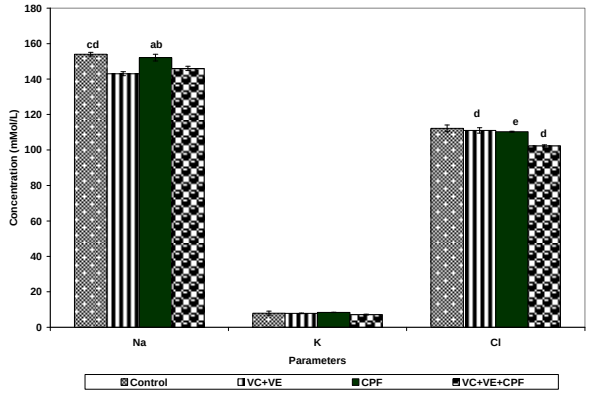

Figure 7: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum electrolytes. [a] $p < 0.05$ versus vitamin C + vitamin E group; [b] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group; [c] $p < 0.01$ versus vitamin C+vitamin E group; [d] $p < 0.01$ versus vitamin C+vitamin E+ chlorpyrifos group; [e] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group; [f] $p < 0.01$ versus vitamin C+vitamin E+ chlorpyrifos group.

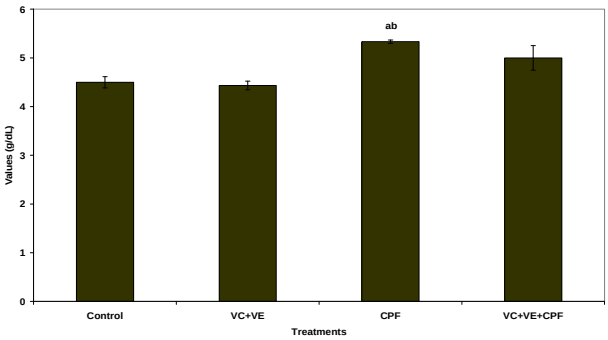

Figure 8: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on total protein concentration. [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C+vitamin E group.

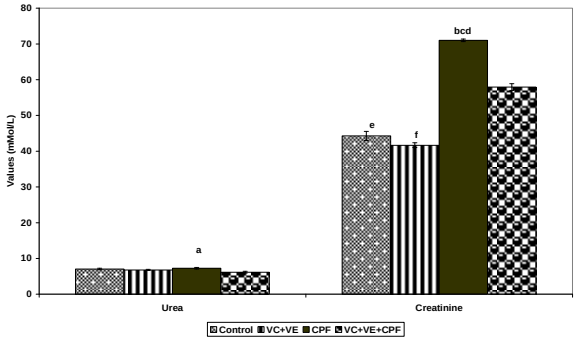

Figure 9: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum urea and creatinine concentration. [a] $p < 0.05$ versus vitamin C+ vitamin E + chlorpyrifos group; [b] $p < 0.01$ versus control; [c] $p < 0.01$ versus vitamin C + vitamin E group; [d] $p < 0.01$ vs vitamin C + vitamin E + chlorpyrifos group; [e] $p < 0.01$ versus vitamin C + vitamin E group; [f] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group.

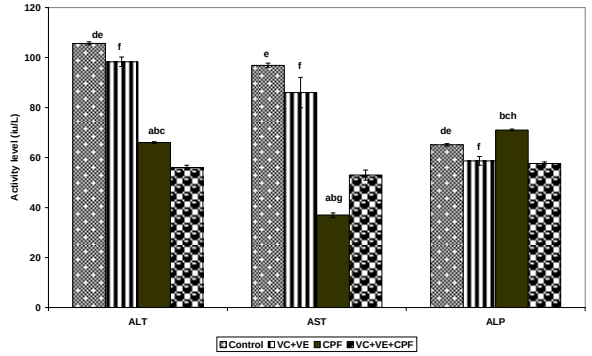

Figure 10: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum enzymes concentration. [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C + vitamin E ; [c] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [d] $p < 0.01$ versus vitamin C + vitamin E group; [e] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [f] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [g] $p < 0.05$ versus vitamin C + vitamin E + chlorpyrifos group; [h] $p < 0.05$ versus control.

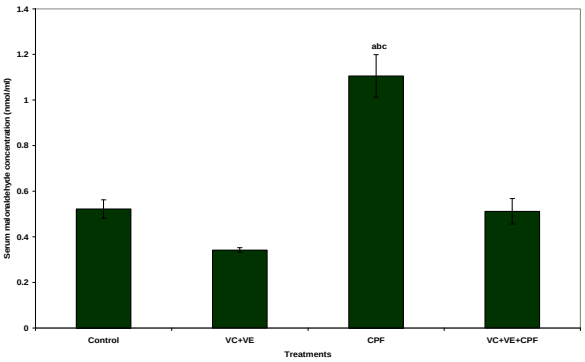

Figure 11: **Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum malonaldehyde concentration.** [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C + vitamin E ; [c] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group.

Discussion

The study also showed that prolonged CPF administration did not cause significant change in body weight of mice. A mere 3% increase in weight gain over a period of 10 weeks demonstrated the adversity caused by prolonged CPF exposure. This finding was in accord with the results obtained in earlier studies[8, 21-23]. However, this result contravened those recorded in other studies[24,25], which showed a dose-dependent decrease in body weight of mice treated with CPF. In the present study, the low weight gain observed in the CPF group compared to what was observed in the other groups despite normal feed consumption may have resulted from alteration in food utilization, perhaps through interaction with enzymes and hormones essential for normal metabolism. Chronic CPF exposure has been shown to cause vacuolization of *zona fasciculata*[25,26], therefore, altering the elaboration of cortisol, which plays essential role in metabolism. Similarly, CPF exposure has been associated with hypothyroidism[8,27], resulting in alteration of metabolic rate. Furthermore, CPF-oxon, the active metabolite of CPF has been shown to inhibit cholesteryl-ester hydrolase, which is an enzyme essential in the promotion of normal reaction of the body to stress[28]. The combination of these effects may have caused mild body weight gains observed in the CPF group. On the contrary, pretreatment with combination of vitamins C and E resulted in a consistent increase in weight gain throughout the study period, and the weight recorded at termination was significantly higher than what was obtained at the commencement of the study. Although the 20% increase in weight gain obtained in the vitamin pretreated group was not as high compared to 33% recorded in the control, it is much higher than those observed in the CPF group. This showed that oxidative stress is an essential mechanism involved in CPF-induced adversity on body weight.

The clinical signs observed in the CPF groups were consistent with cholinergic symptoms observed in OP poisoning. These symptoms resulted from the AChE inhibition caused by CPF and its active metabolites, CPF-oxon, leading to accumulation of acetylcholine in the cholinergic receptors [3]. The mitigation of clinical signs and mortality in mice pretreated with vitamins C and E demonstrated the protective effect of the vitamins on CPF-induced toxic signs and death. This shows the role of oxidative stress in toxic signs evoked by CPF. Apart from its direct effect on free radical, vitamins C and E have been shown to partially restore the activity of AChE[20], which may have contributed to the mild toxic signs observed in the vitamins pretreated groups.

The significant elevation of PCV, Hb concentration and RBC counts in mice administered CPF only may be due to the mild diarrhea and the resultant hemoconcentration. However, vitamins C + E pretreatment significantly suppressed the adverse hematological effect by CPF. The leukopenia observed in the CPF group showed its immunotoxic potentials. The neutropenia in the CPF group may be related to the essential role played by neutrophil in free-radical mediated injury by inducing extracellular release of superoxide and other free radicals[29]. This also leads to neutrophil destruction resulting in their decrease in the peripheral circulation. Pretreatment with combination of vitamins C and E significantly improved the concentration of leukocytes in the circulation, indicating that oxidative stress plays an essential role in the leukopenia induced by prolonged CPF administration. Vitamin E is an essential intracellular antioxidant in the cytomembranes responsible for the maintenance of cellular integrity[30]. Therefore, the membrane stabilization by vitamin E may have played a significant role in the improvement of the cellular integrity of the neutrophils, preventing the release of the cell damaging free radicals. Similarly, vitamin C may have assisted in this role by scavenging for free radical in the extracellular medium, and regeneration of active vitamin E.

Exposure to prolonged CPF exposure did not significantly alter the Na^+ concentrations compared to the control, despite the mild diarrhea provoked by the insecticide. However, pretreatment with the vitamins lowered the Na^+ concentration significantly compared to the CPF group. Prolonged CPF administration did not significantly alter the serum level of K^+. Similar to what was observed with Na^+, pretreatment with vitamins did significantly reduce the K^+ concentration compared to the CPF group. CPF exposure did not also significantly alter the serum Cl^- concentration compared to the control. On the contrary, pretreatment with the vitamins significantly lowered the Cl^- concentration compared to the CPF group. The reason for decrease in Na^+, K^+ and Cl^- concentrations in the two groups administered vitamins C and E compared to the CPF group is unknown. The increased TP concentration in the CPF group may have been due to hemoconcentration, resulting from the mild diarrhea. Pretreatment with the vitamins did ameliorate the high TP concentration resulting from CPF exposure.

The increased urea concentration in the CPF group showed that the insecticide caused pathological changes in the liver. The reduced urea concentration in group pretreated with vitamins C and E was an indication of their protective effect in CPF-induced lipoperoxidative damage to the liver. Similarly, the high creatinine concentration evoked by prolonged CPF exposure was ameliorated by combination of vitamins C and E. This showed that the antioxidant vitamins protected the kidneys from the lipoperoxidative changes provoked by CPF. Pretreatment with a combination of vitamins C and E did significantly

reduce the creatinine level compared to those observed in the CPF group. This showed that the vitamins protected the kidney from damages provoked by CPF, probably due to their free radical scavenging ability.

The low ALT and AST activity in mice exposed to prolonged CPF agreed with the previous findings[8,25]. Currently, the toxicological significance of low ALT and AST activities is not known. However, pretreatment with the vitamins resulted in a significant improvement in the level of AST but not ALT, which was further lowered. The high ALP activity in mice exposed to prolonged CPF indicated pathological changes in the organs such as the liver, skeletal muscles and bones producing this enzyme. The significant reduction in ALP activity in group pretreated with vitamins C and E demonstrated their protective effect on CPF-induced tissue damage, probably as a result of their antioxidant effect. Studies have shown that CPF causes damage to the liver[6,8, 31,32]. It has been demonstrated that pesticide mixture including CPF induced 8-OH-2-deoxyguanosine in the liver of rat, indicating free radical DNA damage[33]. CPF has been shown to impair antioxidant enzyme activities either directly or through the induction of free radicals [4,34], resulting in oxidative stress. Therefore, the ameliorative effect of vitamins C and E on serum enzymes activities reaffirmed the role of oxidative stress in CPF-induced organ damage and the protective effect of antioxidant vitamins.

The increased serum MDA concentration observed in CPF group indicated that the insecticide evoked lipoperoxidative damage to the tissue through free radical induction. This findings agreed with results obtained in the previous studies[8,33, 35-37].Tissue lipid peroxidation is a degradative phenomenon as a consequence of free radical chain production and propagation which affects mainly polyunsaturated fatty acids[38]. The significantly low MDA concentration in vitamins pretreated group showed their ability to quench CPF-induced tissue lipoperoxidative damage. This may have been responsible for amelioration of the CPF-provoked clinical, hematological and biochemical deficits. Vitamins C and E have been shown to act synergistically as antioxidants[39-40]. Vitamin E acts in the lipid component of the membrane to prevent lipid peroxidation, whereas vitamin C is hydrophilic and an important antioxidant in the biological fluid[41]. Vitamin C also has a sparing effect on vitamin E by facilitating the regeneration of α- tocopherol[42-43]. Furthermore, vitamins C and E have been shown to restore the decreased activities of the antioxidant enzymes, superoxide dismutase and catalase, caused by CPF-ethyl[4] thereby boosting the body's antioxidant reserve. Apart from its antioxidant effect, other non-antioxidant related effect of the vitamins may have been involved in the tissue protective effect observed in the present study. Vitamins C and E have been shown to increase the activity of paraoxonase[44], which is involved in the detoxification of OP compounds. Furthermore, vitamin C is known to serve as cofactors in many essential enzymes involved in metabolism[45,46].

In conclusion, the present study has shown that oxidative stress plays an essential role in CPF-mediated injury and the combination of vitamins C and E ameliorated the injury through its free radical scavenging effect. Therefore, the administration of both vitamins C and E may be of value to farmers and other workers who are frequently exposed to CPF in reducing tissue injury mediated by this OP compound.

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Phytotoxic and Anti-microbial activities of Flavonoids in *Ocimum gratissimum*

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Abstract

In this study, the leaves of *Ocimum gratissimum* have been investigated and found to contain flavonoids as part of their secondary metabolites. This observation agrees with the few available reports on the presence of flavonoids in *O.gratissimum* plants. The flavonoids were extracted from dried powdered leaves of *Ocimum gratissimum* using soxhlet extraction method. The crude extract was partially purified on column chromatography using an eluting system of formic acid and ethyl acetate in the ratio of 15:85.Qualitative tests were carried out to confirm the presence flavonoids in the O. gratissimum extract. Frothing test, as a follow up was also done on the extract to ensure the absence of saponins which are usually abundant as secondary metabolites in plants. The allelopatic investigation of the partially purified extract on bean and maize seeds germination, and on seedlings growth showed that *O.gratissimum* flavonoids are phytotoxic. The inhibition of the radicle and coleoptile growth was observed to be dose-dependent, and the radicles of both seeds were comparatively more inhibited. Eight human pathogenic microbes; six bacteria and two fungi were used to evaluate the antimicrobial activities of the flavonoid extract. A broad-spectrum antimicrobial effect was observed with the flavonoids. However, they had no effect on fungi growth. [Life Science Journal 2010;7(3):45-48]. (ISSN: 1097-8135).

Key words: *Ocimum gratissimum*, flavonoids, allelopatic, phytotoxic, pathogenic microbes, antimicrobial.

INTRODUCTION

Flavoniods, as established natural anti-oxidant, have captured a fast growing interest among consumers and scientists in medical, pharmaceutical, chemical and agricultural industries. They are a group of polyphenolic compounds diverse in chemical structure and characteristics, found ubiquitously as secondary metabolites or chemical constituents in plants either consumed as foods or used by man in folk-lore medicine. They occur naturally in fruit, vegetables, nuts, grains, seeds, flowers, roots, stems and bark of plants; and are integral part of the human diet (Haslam, 1998, Middleton and Kandaswami, 1993).

Literature reports on the presence of flavonoids and other active chemical substances such as tarnins, terpenes, saponins, xanthones and glycosides as phytochemicals in different tropical plants which serve basically as foods and medicinal herbs abound and are daily on the increase following continuous scientific investigations (Sofowara, 1993). The phytochemicals present in these plants are largely responsible for the medicinal functions associated with them.

However, reports on the presence of flavonoids in *Ocimum gratissimum* are quite scanty. The genus, *Ocimum* with the general name Basil, belongs to the family of plants known as Labiatae. It is a relatively small genus of herbs and semi-woody shrubs. It is popularly called scent leaf because of its characteristic aromatic smell. About thirty species have been reported in tropical and sub- tropical parts of the word (Burkhill, 1985). *Ocimum gratissimum* is a vegetable plant of wide nutritional and medicinal applications in Nigeria and in some other parts of the world. It is therefore expedient to evaluate the bioactivities which underline the nutritional and medicinal relevance of this plant. Consequently, the objective of this work was to investigate and evaluate the plant flavonoids for its phytotoxic and anti-microbial activities.

Materials and Methods

The plant material

Fresh *Ocimum gratissimum* leaves were collected from a vegetable garden in Benin City, Edo State Southern Nigeria. The botanical identification was confirmed at the Herbarium of the Department of Botany, University of Ibadan.

Extraction of flavonoids from the plant material

The leaves of *Ocimum gratissimum* were thoroughly air-dried and were ground into powdery form using a warring blender. Five hundred grammes (500g) of the powdered leaves were put into extraction thimbles and top covered

with cotton plug. These were then extracted with absolute methanol for about 24hrs in a soxhlet apparatus to remove flavonoids and other low molecular weight compounds such as sugars, phenols, saponins and oligosaccharides (Fenwick *et al*, 1992). Prior to this, a preliminary extraction with boiling petroleum ether was carried out for 24hrs to remove lipids and various pigments. The resultant methanolic extract was evaporated to dryness in a pre-weighed beaker and its weight was determined. This extract was observed to be dark-brown in color and aromatic in flavor.

Purification of the crude methanolic flavonoid extract

The crude extract was purified with column chromatography using 250g of silica gel (F_{259} grade), a glass column size of 400mm by 3.5mm and 15:85 mixture of formic acid and ethyl acetate as eluting solvent. The eluent was collected in fractions in test tubes at a constant volume of 20ml per tube. Fifty-four fractions labeled T_1 to T_{54} were further purified with Thin-Layer Chromatography. Aliquots from each of the fifty-four fractions were collected and spotted on TLC plates using capillary tubes. The TLC plates were subsequently developed using a solvent system of formic acid and ethyl acetate in the ratio of 15:85. The developed plates were air-dried for about 30mins, and were then visualized under the UV-spectrophtometer for possible fluorescence at a wavelength of 365nm, characteristic of flavonoid spots.

Test for flavonoids in extract fractions

Two methods were used to determine the presence of flavonoids in the extract (Sofowara, 1993;Harbrone, 1973). 5 ml of dilute ammonia solution were added to a portion of each extract fraction followed by addition of concentrated H_2S0_4. A yellow coloration observed indicated the presence of flavonoids. The yellow coloration disappeared on standing. Few drops of 1% aluminium solution were added to a portion of each fraction. A yellow coloration was observed indicating the presence of flavonoid

Collection of flavonoids

The extract fractions which tested positive to flavonoid test were pooled together in a pre-weighed beaker and then concentrated. The beaker with its content was weighed after evaporation, and the difference gave the approximate weight of the partially purified flavonoids. The concentrate was dark brown slurry. It was on this that the various bioassays were carried out.

Phytotoxic Assay

1% Flavonoids solution was prepared by dissolving 1.0g of the partially purified extract in 100ml of distilled water. Serial dilutions of 0.5%, 0.25% and 0.125% flavonoid solutions were subsequently made.10 sterilized Petri-dishes were divided into two groups of five Petri-dishes for the cultivation of the bean and maize seeds. Clean cotton wool was placed at the bottom of each petri-dish, and those with bean were labeled as group I and those with maize seeds as group II.

Group I: 10mls of each of the Flavonoid solution (1%, 0.5%, 0.25% and 0.125%) were pipette into four correspondingly labeled Petri-dishes so that the cotton wool was well soaked. 10ml of distilled water was introduced into the fifth Petri-dish labeled as control. Four viable bean seeds were then arranged in a circular pattern on each of the five petri-dishes. 10ml of distilled water was added to each petri-dish everyday from the second day of the experiment. This was to make up for water loss through evaporation. The germination and growth pattern were observed for seven days. After this, the root and shoot lengths were measured using a thread and a ruler. The recorded values were compared with those of the control groups to determine the index of inhibition of seed germination and growth. The same procedure was concurrently used for the maize seeds and labeled as group II.

Antimicrobial assay

20 mg/ml flavonoid solution was obtained by dissolving 0.2g of the extract in 10ml of sterile distilled water, and used as the standard extract concentration (*Hirasawa et al, 1999*). 10mg/ml, 5mg/ml, and 2.5mg/ml test solutions were subsequently prepared by serial dilution, using water as the diluents.The culture media used were carefully handled and prepared according to the manufacturer's instruction. They were all commercial products of oxoid Ltd Company, England. Six bacteria (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeriginosa, Proteus mirabilis, Klebsiella spp)* and two fungi (*Candida albicans and Aspergillus niger*) were used for this assay. The antibacterial and antifungal activities of the test sample were done using the agar well diffusion method (Stoke and Ridgeway, 1980). Sterile distilled water used as the control. The inhibitory zones produced were measured in millimeters. Negative results were regarded as those in which no zone of inhibition was observed.

RESULTS AND DISCUSSION

The results of the effect of flavonoids on the growth profile of maize and bean seedlings are presented in Table 1. Significant difference between the shoot/root lengths of the treated bean/maize and control seedlings was observed mainly at flavonoid concentrations of 0.5% and 1.0%. The germination of seeds occurred faster in the control media relative to those treated with flavonoid extract, meaning that the flavonoids from *O.gratissimum* inhibit seed germination. Generally, the bean seeds germinated faster than their maize counterparts. This indicates that the maize seeds were more susceptible to the phytotoxic effects of the Flavonoids. Moreover, the inhibitory effects on the germination of bean and maize seeds and on their radicules and coleoptiles were observed to increase with increase in the concentration of the extract. This implies that the inhibitory activity of the *O.gratissimum* flavonoids is dose dependent.

Tables 1a & 1b show that O.gratissimum flavonoids are phytotoxic even at low concentrations. At concentrations of 0.1%, 0.25%, 0.5% and 1.0% the *O,gratissimum* flavonoids in a dose-dependent pattern reduced the growth profile of both maize and bean seedlings. There was a high indication

that these Flavonoids retarded seed germination and seedling growth by inhibiting certain endogenous growth hormones such as gibberellic acid (GA3), indol acetic acid oxidase (IAA-oxidase) and indo-3-acetic acid (IAA) [responsible for seedling elongation]. Thus, their application in agriculture could be considered in weed control

The *O.gratissimum* flavonoids were effective against all bacteria used, both gram positive and gram negative at dose concentrations of 20mg/ml and 10mg/ml. At 5mg/ml, the flavonoids were only effective against *E.coli* and *Proteus mirabilis* and at 2.5 mg/ml, *E.coli* was the only microbe susceptible to the antimicrobial effect of O.gratissimum flavonoids.(Table 2c).This indicates that *O.gratissimum* flavonoids have a broad-spectrum anti-bacterial activity. They are however not antifungal. The inhibitory effect of the flavonoid extract on the selected bacteria was observed to increase with increased concentration of the flavonoid extract, indicating a dose dependent effect. At 20mg/ml dose concentration, flavonoids in *O.gratissimum* showed a potency range of 58.3% to 85.7% relatively to the various standard antibiotics used as positive control (Table 2c). The antibiotics used as standard (positive control) against the pathogenic organisms are standard orthodox drugs used for the treatment of infections in which the pathogens are implicated (Table 2a). Moreover, the bacteria against which the *O.gratissimum* flavonoids were effective are pathogens already implicated in the etiology and severity of human diseases. Thus, these flavonoids may probably be of immense potential application in pharmaceutical and medical formulations. The possibility of further purification and formulation of these flavonoids into antibiotics should be considered.

Table 1a: Effect of *O.gratissimum* Flavonoids on the growth profile of bean and maize seedlings.

Conc. of Flavonoid extract	ASL (cm), Beans	ASL (cm), Maize	ARL (cm), Beans	ARL(cm), Maize
0.10%	8.02	7.45	5.87	5.22
0.25%	7.01 [b]	6.77	5.12 [b]	4.53
0.50%	6.08 [b]	5.81 [b]	4.01 [a]	3.84 [b]
1.00%	4.06 [a]	3.98 [a]	2.52 [a]	2.41 [a]
Control	8.86	7.91	7.20	5.93
a= significant difference ($p<0.05$) against control	b= significant difference ($p<0.01$) against control			

ASL= Average shoot length, ARL= Average root length. *Values are average results of six sets of the experiment*

Table 1b: Percentage inhibition of root/shoot growth profile by O.gratissimum fllavonoids.

Conc. of Flavonoid extract	% inhibition of shoot, Beans	% inhibition of shoot, Maize	% inhibition of root, Beans	% inhibition of root, Maize
0.1%	9.48	5.80	18.47	11.17
0.25%	20.88	14.4	28.89	23.60
0.5%	31.38	26.50	44.31	34.73
1.0%	54.17	49.70	65.00	59.36

Table 2a: Pathogenic organisms and selected standard antibiotics (+ve control)

Pathogenic organism	*S. aureus*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*P.mirabilis*	*Klebsiella spp.*	*A. niger*	*C.albicans*
Standard antibiotic	Gentamycin			Tetracycline			Nystatin	

Table 2b: Diameters (mm) of inhibition zones of microbial growth by O.gratissimum flavonoid extract

			Conc. of Flavonoid extract (mg/ml)			
Organism	**-ve control**	**+ve control**	**2.5**	**5.0**	**10.0**	**20.0**
Staphyococcus aureus.	0	22	0	10	14	18
Escherichia coli	0	28	12	15	18	24

Bacillus subtilis	0	20	0	0	10	14
Pseudomonas aeruoginosa	0	18	0	0	10	12
Proteus mirabilis	0	24	0	28	12	16
Klebsiella spp.	0	23	0	0	12	15
Aspergillus niger	0	22	0	0	0	0
Candida albicans	0	16	0	0	0	0

Values are mean results of six sets of the experiment

Table 2c: Percentage (%) inhibition of microbial growth by O.gratissimum extract

	Conc. of Flavonoid extract (mg/ml)			
Organism	**2.5**	**5.0**	**10.0**	**20.0**
Staphyococcus aureus.	0	45.5	63.6	81.8
Escherichia coli	42.9	53.6	64.3	85.7
Bacillus subtilis	0	0	50.0	70.0
Pseudomonas aeroginosa	0	0	55.6	66.7
Proteus mirabilis	0	30	50.0	58.3
Klebsiella spp.	0	0	52.0	65.2
Aspergillus niger	0	0	0	0
Candida albicans	0	0	0	0

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5/20/2010

Mycological, Biochemical and Histopathological Studies on Acute Fusariotoxicosis In Sheep.

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ABSTRACT: One hundred cases of diseased sheep at desert districts in governorates of (Giza; 6th. October and El-Wadi-El-Gadid), were investigated. Sixty percent of these sheep sera had a mean levels of T-2, zearalenone and fumonisins (2.5±0.2, 4.3±0.5 and 25.0±2.0) respectively. The used feeds and underground water in breeding of this sheep were examined mycologically which revealed that all examined samples gave a variable rates of pollution. Seven genera and 15 species of fungi were recovered from feeds and water. The most predominant isolates belong to members of genus Aspergillus with a range of (5-100%), followed by Fusarium spp. with a range of (40-90%), Penicillium spp. with a range of (10-55%) and Mucor spp. with a range of (10-50). The Fusarium toxins were detected in same feed samples, the largest amount estimated in crushed yellow corn (60%) namely FB1, T2 and zearalenone with the mean levels of (48.4±1.0; 3.0±0.1 and 0.84±0.03) respectively. The significant high levels of FB1 in the present feed samples and serum of diseased sheep gave a large possibility that FB1 was responsible for this disease outbreak in sheep. On the other hand, the biochemical examination of diseased sheep sera for estimation of toxic effects is based on the assumption that the elevated activities in levels of serum enzymes such as (AST, ALT, GGT, LDH and urea). While, slightly decreases in ceratinine, calcium and phosphorus levels compared with the apparently healthy group. The pattern of protein electrophoresis showed a significantly decreased values in serum total protein, alpha globulin, beta globulin and while slightly increase in gamma globulin. The internal organs of dead cases during this disease had various significant pathological changes in vital organs including hemorrhagic, alveolar pneumonia and calcification in lung. The liver showed hemorrhage, oedema, vacuolar degeneration and necrosis of hepatocytes with evidence of preneoplastic stage in liver cells. Whereas, the kidney showed vacuolar degenerating changes and necrosis of the tubular epithelium, in addition to glomurular oedema and calcium deposition. This study increased awareness of the significant dangerous effect of environmental pollutions particularly fusarium species and their toxins. This study increased awareness of the significant dangerous effect of environmental pollutions particularly fusarium species and their toxins. [Life Science Journal 2010;7(3):49-57]. (ISSN: 1097-8135).

Keywords: pollution; biochemical alterations; fusarium

INTRODUCTION

The increased importance of animal production due to progressive elevated requirement of human consumption gave an intensive attention of animal health status. The environmental pollution is considered the essential cause of animal diseases particularly pollution with fungi and their toxins for the used feed and water in animal breading and elsewhere, contamination of human food. Mycotoxins are a group of structurally diverse, mold elaborated compounds that induce diseases known as mycotoxicosis in humans and animals. As much as twenty-five percent of the world's food crops are estimated to be contaminated with mycotoxins. Ingestion of sufficient quantities of mycotoxin-contaminated material leads to acute, and more commonly, chronic intoxication ***(Hassan et al., 2003; and 2009).*** The mycotoxins of greatest agricultural and public health significance include aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone, and ergot alkaloids ***(Hassan et al., 2004; 2008 and 2009).*** However, the fungi of *Fusarium species* and their toxins are widely distributed through the world where they occur in soil, on plants, plants debris and similar organic subtracts. They cause significant economic losses in agriculture, morbidity and mortality in animals and immunological compromised humans, where it is capable of killing cells by causing extensive damage to cellular membrane **(*Ajello and Hay, 1998 and Mogeda et al., 2002).*** On the other hand, epidemiological studies associated with fusarium toxins had a wide range of biological effects, including pulmonary oedema in pigs and ruminants **(Harrison et al., 1990),** nephrotoxicity and liver cancer in rats **(Gelderblom et al., 1996).** Although, its effects on human are difficult to be determined. Fumonisin B9 had been statistically associated with a high incidence of oesophageal cancer in certain areas of Transkei, South Africa and also in China ***(Chu and Li, 1994).*** The International Agency for Research on Cancer has declared *F. moniliforum* form toxins as potentially carcinogenic to human. ***Gelderblom et al. (1994)*** proposed that FB1 was a tumour promoter at doses not causing significant liver pathology but when given at overtly hepatotoxic dose, it was also a weak initiator. Also, the lymphocytes decreased in response to Zeraralonone especially for LD50 dose. Many data showed that this mycotoxin induced immunosupression in depressing T or B lymphocyte activity ***(Berek et al., 2001).*** All the previous literatures recorded that the pollution affect upon the growth rate and health of human being and animals including aneamia , stunted growth , carcinogenic, tremorgenic, haemorrhagic, dermatitic, pulmonary edema, immunosuppressive and hormonal effects ***(Hassan, 1998 and 2003 ;and Hassan et al., 2003 ;2004;2008 and 2009)***. Whenever, sheep breeding and their production is the main source of food for human in the desert districts. So, the aim of the present

work was to investigate the problem of fungal and fusarium mycotoxins pollution of feed and underground water and its role in the health status of sheep at some deserts Governorates (Giza, El-Wadi El Gadid and 6 th October).

MATERIAL AND METHODS

Material:

Samples:

Serum, feed and water samples: One hundreds diseased cases of sheep at desert districts in governorates of Giza; 6th October and El-Wadi-El-Gadid were investigated. The cases of sheep suffered from loss of weight gain, low productivity, diarrhea, mastitis, disturbance in fertility and sudden mortality of some cases. From districts of diseased cases, 100 samples of sera, 150 feeds and 20 samples of underground water which used in breeding of diseased sheep were collected. The samples of feed and water were collected in sterile plastic container to prevent any contamination.

Internal organs: From the recently deed cases of animal from disease outbreak, the internal organs were collected and imbedded in bottles containing 10% formalin solution for further histopathological examination. These organs included liver, kidney, lung, bronchial lymph node and heart.

***Mycotoxins standards*:** Standers and immunoaffinity column of Zearalenon, T2 and FB1, were purchased from Sigma Chemical Company (USA).

Methods:

Mycological examination of samples:

The samples of feeds and underground water which used by symptomatically diseased sheep cases were subjected for isolation and identification of fungi as recommended by ***(Conner et al., 1992)*.**

Detection of mycotoxins in feed and sera of diseased sheep:

Detection of mycotoxins in serum of sheep and feed stuffs by fluerometric methods as described by ***Hansen (1993)*** using immune-affinity column method**.**

Biochemical investigations of sheep sera:

From each of investigated animal a blood samples were collected in small labeled dry and clean vials without anticoagulant in centrifuge tube, allowed to clot and then centrifuged at 3000 rpm for 90 minutes for separation of serum which used to assay the biochemical parameters The biochemical assays of serum gamma glutamyle transferase (GGT) and lactic dehydrogenase (LDH) activities were determined according to methods of **(Szase et al., 1976)** ,aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities according to ***Reitman and Frankel, (1957),*** serum urea according to **Wybenga *et al.* (1971**), serum creatinine level according to ***Henry (1974)***, Estimation of serum total protein and electrophoretic pattern were carried out after ***SonnenWirth and Jaret (1980) and Davis (1964),*** respectively.

However, measurement of calcium, ph. and Mg. were carried out as the technique described in the references ***(Brown et al., 1986 and Brown and Taylor, 1995)***.

Histopathological studies:

From the recent dead cases, tissue specimens were collected directly from lung, bronchial lymph node, heart, liver, spleen, kidneys and intestine for histopathological examination. They were kept in 10% neutral buffered formalin for at least 24 hours, routinely processed by the standard paraffin embedding technique and stained with Hematoxylin and Eosin. Prussian blue stain was used for hemosidrin pigments staining ***(Bancroft et al., 1994).***

STATISTICAL ANALYSIS: The obtained date were computerized and analyzed for significance, Calculation of standard error and variance according to ***(SPSS 14, 2006)***.

RESULTS AND DISCUSSION

The economical importance of sheep animals in desert districts Governorates were at the top to other part in Egypt, Where, peoples in these districts their life depend on its products such as meat, milk, wool and leather obtained from these animals ***(Agaoglu, 1991; Camas et al., 1994* and *Hassan et al., 2008***)

In this paper, the current data in table (1) showed that, sera of one hundred cases of diseased sheep outbreaks which suffered from loss of weight gain, low productivity, diarrhea, mastitis, disturbance in fertility and sudden mortality of some cases at desert districts in governorates of Giza; 6thOctober and El-Wadi-El-Gadid, contained significant levels of fusarium toxins. Meanwhile, sixty percent of these sheep had the mean levels of fusarium toxins as T-2, zearalenone and fumonisins (2.5±0.2, 4.3±0.5 and 25.0±2.0) respectively. .The results indicated that serum of diseased sheep contained higher mean significant level of FB1 than other types of fusarium toxins which suggested being the essential cause of disease. Mycotoxins in sera of sheep and cattle in Egypt in association with symptoms of toxicities were previously reported by ***Hassan (1994); Hassan et al. (2003; 2004 and 2009)***.

Table (1): Determination of fusarium toxins in serum of diseased sheep .

Animals	Prevalence of fusarium toxins			Mean levels of fusarium toxins (ppm)		
	No. of tested	No. of +ve	%	Fumonisins	T-2	Zearalenone
Sheep	100	60	60	25.0±2.0	2.5±0.2	4.3±0.5

The effects of fusarium toxins in human and animals ranged from carcinogenic and nephrotoxic and immunosuppressive health effects (**Morriss, 1997)**. Although the main route of human exposure to mycotoxins has been identified as the direct ingestion of contaminated cereals and grains **(Morriss, 1997)**, while, there are many studies about whether the ingestion of meat, milk, and eggs originating from mycotoxin-exposed food-production animals is a significant exposure pathway for mycotoxins among humans **(Hassan et al., 1997; Wafia and Hassan, 2000 and Hassan et al., 2004 and 2009).** The search focused to recovered the accurate causes and sources of this disease in sheep, therefore, the direct factors to the animal consumption were examined .The fungal examination of feeds , feedstuffs and underground water (which the only available source of water in these districts), the results revealed that all examined samples gave a variable rates of pollution. Seven genera and 15 species of fungi were isolated from feeds and water. The most predominant isolates belong to members of genus Aspergillus with a range of (5-100%), followed by Fusarium spp. with a range of (40-90%), Penicillium spp. with a range of (10-55%) and Mucor spp. with a range of (10-50%). Whereas, the frequency of isolation of other spp. as Rhizopus spp., C.albicanse and Rhodotorula spp. were relatively low. On the other hand, the fungal contamination of underground water was significantly high as compared with standard healthy water which must be free from any signs of pollution (Table, 2). However, F.moniliform, F.oxysporum and F. solani were the most frequent isolated members of Fusarium from feed samples (Table, 3). The fungus of F.moniliform was recovered from all examined feed samples at a rates ranged from (20-65%), while, F.oxysporum was isolated from lower examined samples (5-10%) with exception of wheat straw samples .Whereas, the species of F. nival and F. fusaroides were only isolated from (Soya bean meal and crushed yellow corn), respectively with the same rate (5%). It is clear from the result that crushed yellow corn and wheat straw were the most contaminated followed by hay, Soya bean and drawa. While, the underground water was the lowest contaminated samples. These differences in the level of contamination may be due to the exposure of the examined samples to different climatic condition either during preparation or transportation or storage. These findings were in agreement with the results of (**Hassan et al. 2003; 2004; 2008 and 2009)**, who recovered most of these fungi from the examined feed and water samples.

Table (2): Prevalence of fungi in feeds and underground water used for breeding of sheep

Fungal Species	Crushed yellow corn(30)		hay(35)		Wheat straw(20)		Soya bean meal(35)		Drawa (Leaves of yellow corn) (30)		Underground water (20)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Aspergillus sp.	20	100	19	95	20	100	15	75	10	50	1	5
A. flavus	18	90	17	85	18	90	7	35	40	20	1	5
A. niger	16	80	15	75	15	75	14	70	36	18	10	50
A. candidus	1	5	--	--	--	--	2	10	30	15	0	0
A. fumigatus	4	20	7	35	--	--	2	10	20	10	1	5
A. ochraceus	5	25	19	5	1	5	1	5	16	8	0	0
A. terrus	5	25	2	10	3	15	3	15	10	5	0	0
Fusarium sp.	10	50	18	90	15	75	8	40	8	40	0	0
Penicillim sp.	7	35	9	45	6	30	10	50	11	55	2	10
Mucor sp.	10	50	6	30	2	10	10	50	3	15	0	0
Rhizopus sp.	1	5	1	5	3	15	4	20	1	5	0	0
C.albicanse	2	10	0	0	0	0	1	5	2	10	1	5
Rhodotorula sp	1	5	0	0	1	5	0	0	2	10	2	10

When, the feed samples which contaminated with fusarium spp. were subjected for detection of Fusarium toxins, the results revealed that the largest amount was detected in crushed yellow corn (60%) namely FB1, T2 and zearalenone with the mean levels of (48.4±1.0; 3.0±0.1 ppm and 0.84±0.03 ppm)**,** respectively.

Table (3): Prevalence of fusarium species in feeds of sheep suffering from problems of animal diseases.collected from different districts at el Wadi El Gedid

Fusarium Species	**Crushed yellow corn**		**Hay**		**Wheat straw**		**Soya bean meal**		**Drawa (Leaves of yellow corn)**	
	No.	%	No.	%	No.	%	No.	%	No.	%
F.moniliforme	4	20	13	65	8	40	6	30	7	35
F.oxysporum	1	5	1	5	-	-	1	5	2	10
F.solani	1	5	1	5	4	20	-	-	-	-
F.sporotrichoides	1	5	-		1	5	-	-	-	-
F. aquaeductum	1	5	-	-	1	5	-	-	-	-
F. nival	-	-	-	-	-	-	1	5	-	-

F. fusaroides	1	5	-	-	-	-	-	-	-	-
F. equiseti	-	-	-	-	1	5	-	-	-	-
F. tricinctum	1	5	3	15	-	-	-	-	-	-

It was interesting to report here that the samples of wheat straw contained only FB1 at a rate of (70%) with a mean level of (20±0.9 ppm) (Table, 4). The significant levels of FB1 in the present feed samples and serum of diseased sheep gave a large possibility that FB1 was responsible for the disease outbreak in sheep**.**

Table (4): Detection of fusarium toxins in feeds .

Fusarium Species	**Prevalence of fusarium toxins**			**Mean levels of fusarium toxins (ppm)**		
	No. of tested	**No. of +ve**	**%**	**Fumonisins**	**T-2**	**Zearalenone**
Crushed yellow corn	10	6	60	48.4±1.0	3.0±0.1	0.84±0.03
Hay	10	5	50	17.0±1.3	-	0.71±0.0
Wheat straw	10	7	70	20±0.9	-	-
Soya bean meal	10	4	40	15.0±0.2	2.0	0.99±0.005
Drawa (Leaves of yellow corn)	10	4	40	27.0±3.22	1.0±0.01	1.50±0.0

The Food and drug administration has established recommended maximum levels for aflatoxins and fumonisins in animal feed. For swine, ruminants including sheep, and poultry, the recommended maximum levels of total fumonisins in complete feeds are 10, 30, and 50 μg/g, respectively **(FDA, 1994).** Therefore, the detected levels of FB1 were significantly over the permissible limits in feeds particularly FB1 toxin in examined sheep feed samples which ranged from (15.0±0.2-48.4±1.0 ppm). The same findings were detected by many authors as **(Hassan et al., 2002; 2003; 2004 ; 2008 and 2009) ; El-Hamaky, 2001 and El Ahle et al., 2006).**

On the other hand, the biochemical examination of diseased sheep sera for estimation of toxic effects is based on the assumption that the elevated activities in levels of serum enzymes such as (AST, ALT, GGT, LDH and urea) in Table, (5). While, a slightly decreases in ceratinine level compared with the apparently healthy group. These results reflect organs damage **(Cheng et al., 2001 and Asrani, et al., 2006).** The increased serum enzymes activity observed by feeding toxic diets in this study may be due to hepatic degeneration and subsequent leakage of enzymes into circulation. **(Chen** et **al., 2008 and Wang et al., 2008).** It is reported that the significant effect of fusarium toxins are the alteration in serum concentration of kidney and liver enzymes ,total protein, albumin, minerals and lipid profiles **(Kubena et al., 1997 and Mogeda et al., 2002).** The high concentrations of serum urea in sheep fed contaminated diet may be a result of increased ammonia absorption caused by altered protein turnover in the rumen micro-flora, or altered protein metabolism in sheep tissues. In ruminants, serum urea levels are affected by protein digestion and metabolism by the rumen biomass. A large portion of dietary protein is hydrolyzed and deaminated by rumen micro-flora, giving rise to peptides and free ammonia in the rumen **(Herdt, 2000).** A portion of the free ammonia is absorbed and is metabolized to urea in the liver. If microbial protein synthesis in the rumen is inhibited by mycotoxins, more free ammonia remains in the rumen, is absorbed into the blood, and is metabolized to urea, resulting in elevated blood urea concentrations. **Danicke et al. (2005)** observed that postprandial rumen fluid ammonia concentrations were consistently higher when *Fusarium* mycotoxin-contaminated wheat was fed to sheep**.** Inhibition of protein synthesis results in elevated concentrations of free Amino acid that are used for energy utilization, resulting in increased serum urea. The results of this study are in agreement with those of **Chowdhury and Smith (2004),** who observed that excessive serum concentrations of uric acid in laying hens were a result of feeding feedborne *Fusarium* mycotoxins. Moreover, in a subsequent study with laying hens, they found that feeding contaminated grains led to reduced hepatic fractional protein synthesis rates **(Chowdhury and Smith, 2005). Danicke et al. (2006)** also observed a reduction in fractional protein synthesis rates in the kidneys, spleen, and ileum of pigs exposed to DON.

At the same time concentrations of serum calcium and serum phosphorus were decreased due to feeding *Fusarium* mycotoxin-contaminated diets This resultes were agree with **Díaz and Smith (2006).**

Fusarium inducing significantly decreased values in serum total protein, alpha globulin, beta globulin and while slightly increase in gamma globulin, these results agree with **(Rotter et al., 1994**).

The globulin component (Table, 6) showed drop in α1, α2 and β2 globulin in all the experiment while decrease γ1 globulin. This may be attributed to that *Fusarium* fungi cause's hepatotoxic, nephrosis, hemorrhages (liver and kidneys) ***(Tietz, 1996)*** *Fusarium* mycotoxins might affect

the synthesis of globulins of hepatic origin as well as globulins of lymphoid origin. **Rotter et al. (1994)** suggested that *Fusarium* mycotoxins can directly affect α-globulin synthesis in the liver. In addition, *Fusarium* fungi has immunosuppressive effect inhibit nearly cellular and humeral immunologic reaction have been reported by **Rocha et al. (2005) i**ncluding disruption of normal cell function by inhibiting RNA, DNA, and protein synthesis; inhibition of cell division; stimulation of ribotoxic stress response; and activation of mitogen-activated protein kinases. It has been found that T-2 toxin is a potent member of the trichothecene group of mycotoxins produced by *Fusarium* fungi **(Bamburg et al.,1970).** It has been found that T-2 toxin is a mycotoxin with immunomodulatory activity, where it can stimulate (immune-stimulation) or inhibit (immune-suppression) the activity of the immune system **(Shinozuka et al., 1997and Pestka et al., 2004)].**

Table (5); Biochemical parameters in serum of diseases sheep cases at desert districts in comparison to healthy cases.

Parameter	Apparently healthy	Diseased group
AST u/l	53.67±4.91	124.9***±7.94
ALT u/l	40.66±2.18	93.6***±5.48
GGT u/l	97.57±1.38	111.56*±5.11
LDH u/l	718.4±22.36	811.0*±24.11
urea mg%	41.11±2.15	53.52**±3.81
Creatinin mg%	1.31±0.07	0.9±0.24
Uric acid mg%	3.17±0.37	5.1**±0.34
Calcium mg%	9.22±0.33	7.46**±0.41
Phosphorus mg%	6.31±0.32	5.77±0.17

Results are expressed as means ± SEM (n =15), student 't' test

To give complete idea about the effect of this disease in sheep, the internal organs of dead cases during disease outbreak in the same desert districts were subjected for histopathological studies. The results revealed that thickening of the pleural membrane was observed with infiltration of mononuclear inflammatory cells, hemorrhage and proliferation of the epithelial cells lining bronchioles. Moreover, in some cases the proliferation was severe and uncontrolled which lead to occluded the bronchial lumen and form nest of epithelial cells with clear eosinophilic cytoplasm giving the feature of preneoplastic stage (Fig. 1, a & b).Some alveoli were filled with red blood cells accompanied with mononuclear inflammatory cells (alveolar pneumonia). Destruction of the wall of some alveoli with infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) were noticed accompanied with hemorrhage, calcification was also detected (Fig. 2, a & b). Severe hemorrhages with infiltration of inflammatory cells with compensatory emphysema (Hemorrhagic pneumonia) were seen in some cases.

While, bronchial lymph node showed moderate to severe depletion of lymphoid follicles, where lymphocytes detected inside alveoli and interalveolar septa in pneumonia. The respiratory tract is the primary rout of entry for Fusarium spp. and their toxins based on the sinopulmonary involvement. It has been speculated that the fusarium toxins produced damage the tissues which allowing the fungus to spread more easily **(Ajello and Hay, 1998).** However**, Halloy et al. (2005) and (Haschek et al., 2001)** mentioned that the lung of experimentally fusariotoxicated piglets particularly with FB1 showed a minimal enlargement of the alveolar septa due to an increase in the macrophage, lymphocyte number and develop lethal pulmonary edema within 4-7 days. Whereas, muscles necrosis and oedema were evident in heart in our study. A various degrees of myocardial degeneration with foci or cellular infiltration and fibrosis were observed in rats with several doses of T-2 toxin, a trichothecene metabolite of Fusarium **(Schoental et al., 1979)**.

Table (6); Patterns of protein electrophoresis in serum of diseases sheep cases at desert districts in comparison to healthy cases (mg/dl).

Parameter	Apparently healthy	Diseased group
Alb	2.35±0.12	1.87**±0.07
T.alpha	0.96±0.1	0.87±0.09
Alpha1	0.41±0.03	0.4±0.02
Alpha1	0.55±0.02	0.47*±0.02
t. beta globulin	1.09±0.04	1.02±0.03
Beta1	0.5±0.02	0.55±0.04
Beta2	0.59±0.01	0.47*±0.04
Gamma1	1.59±0.11	1.53±0.05
Gamma2	0.34±0.03	0.520.03
Gamma globulin	1.93±0.15	2.05±0.1
T.globulin	3.98±0.33	3.940.29
A/G ratio	0.59±0.03	0.43**±0.03
T. protein	6.33±0.55	5.81±0.08

- Results are expressed as means ± SEM (n =15), student 't' test

Many researchers mentioned that fusarium toxins particularly FB1 produces a wide range of biological effects including nephrotoxicity and liver cancer in rats **(Gelderblom et al., 1996).** The present study revealed glissonian's cirrhosis in liver, vacuolar degeneration and necrobiotic changes of hepatocytes in addition to haemorrhages and oedema in between hepatocytes). Some liver cells arranged in irregular aceni (preneoplastic stage) (Fig. 3 a & b). Thickening of the wall of central vein was also noticed. Epithelial hyperplasia of bile duct was detected with the formation of newly formed bile ductules. There were aggregation of oval vesicular cells in the portal area with infiltration of mononuclear inflammatory cells and fibrous connective tissue formation. Similar lesions were illustrated caused by FB1 **(Abbes et al., 2006 and Voss et al., 2001)** and zearalenone **(James and Smith, 1982).** According to data of the **National Toxicology**

Program (USA) (1982), ZEN was found to produce hepatocellular adenoma. While**, Abbes et al. (2006**) mentioned that the histological examination of mice kidney that treated with two ZEN doses alone revealed a swelling in the epithelial cells of the proximal tubules, granular degeneration, shrunken glomeruli with the presence of eosinophilic cast in the lumen of tubules and blood vessels dilatation.

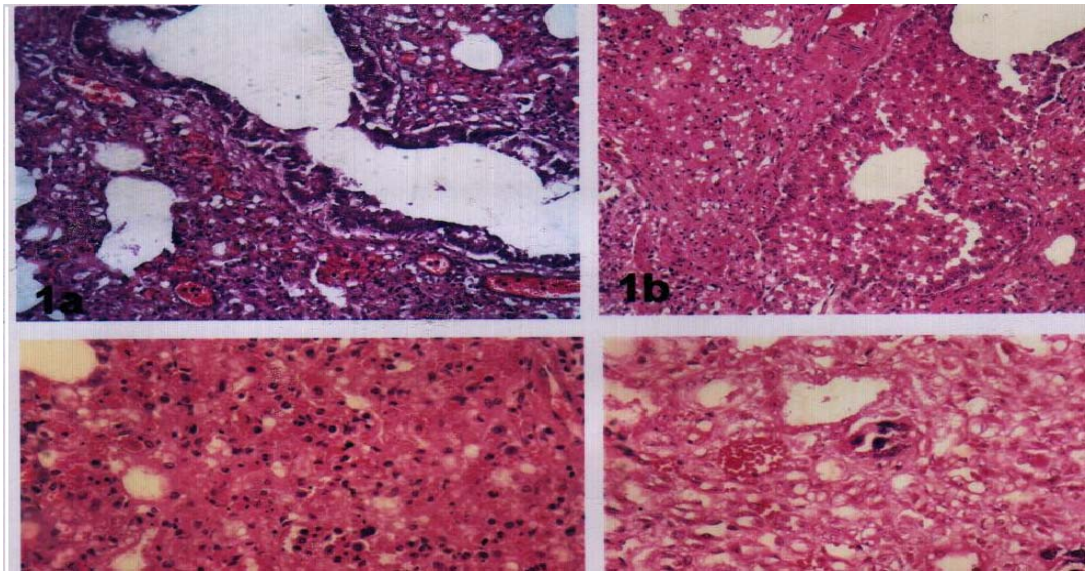

Fig. (1, a & b): Lung of sheep fed on mycotoxin (FB1, T2, ZNE) showing proliferation of the epitheliial cells lining bronchiols was severe, uncontrolled and form nest of epithelial cells giving the feature of preneoplastic stage (H & E X 100).

Fig. (2, a & b): Lung of sheep fed on mycotoxin (FB1, T2, ZNE) showing destruction of the wall of some alveoli with infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) accompanied with hemorrhage and calcification (H & E X a) 200, b) 400).

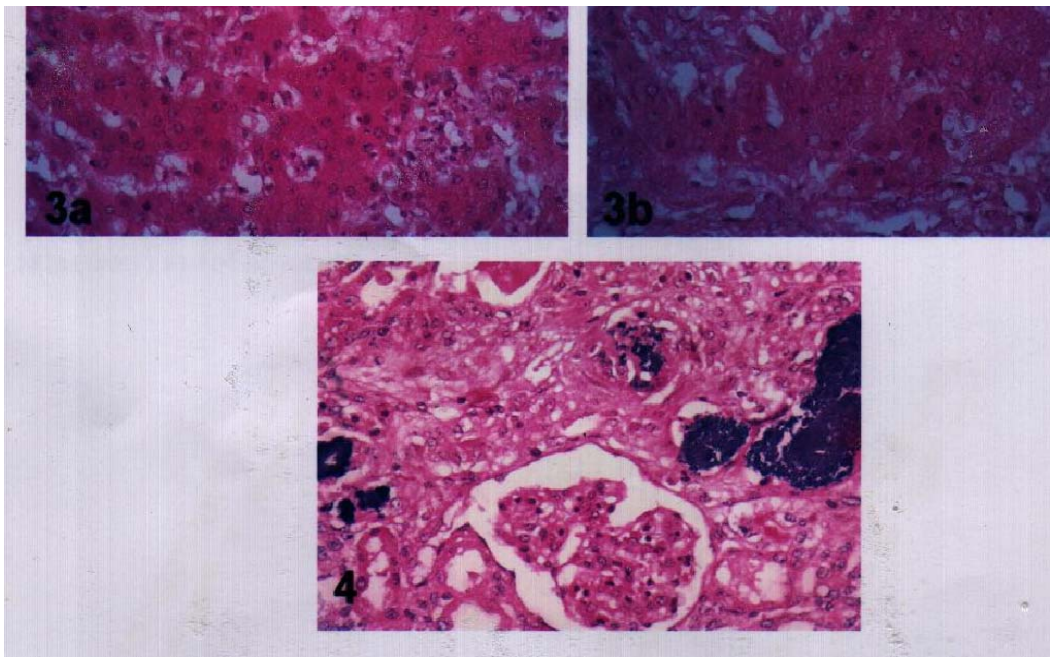

Fig.(3, a & b): Liver of sheep fed on mycotoxin (FB1, T2, ZNE) showing disorganization of hepatic cord (a) with tendency to formation of irregular aceni (preneoplastic stage) (b) (H & E X 400).

Fig. (4): Kideny of sheep feeding on mycotoxin (FB1, T2, ZNE) showing necrosis of renal tubular epithelium, gromerular oedema and calcium deposition. (H & E X 400).

These confirm our results showed in kidney in our study, where, the pathological examination of kidney revealed blood vessels dilatation. Vacuolar degeneration of epithelial cells lining the renal tubules were noticed, other were sloughed in the lumen forming renal casts. Meanwhile, some tubular epithelium revealed necrosis, glomerular oedema and calcium deposition were also detected (Fig.4). ***Voss et al. (2001),*** Mentioned that FB1 induces apoptosis of hepatocytes and proximal tubular epithelial cells. More advanced lesion in both organs is characterized by simultaneous cell loss (apoptosis and necrosis) and proliferation (mitosis). Microscopic and other findings suggest that an imbalance between cell loss and replacement develops a condition favorable for carcinogenesis. On the molecular level, fumonisins inhibit cermide synthase and disrupt sphingolipid metabolism and theoretically, sphingolipid-mediated regulatory processes that influence apoptosis and mitosis.

The previous literatures recorded that the pollution affect upon the growth rate and health of human being and animals including aneamia , stunted growth , carcinogenic, tremorgenic, haemorrhagic, dermatitic, pulmonary edema, immunosuppressive and hormonal effects ***(Hassan, 1998 and 2003 ;and Hassan et al., 2003 ;2004;2008 and 2009)***.These findings were confirmed in our study, where , the above results clearly observed the effects of fungal particularly fusarium species and their toxins in sheep at desert districts.

It can induce both toxicologic and immunotoxic effects in a variety of cell systems and animal species as cytotoxic effect to reticulocytes, fibroblasts and lymphocytes and the cellular toxicity appears to be mediated by the inhibition of protein synthesis as reported by ***(Ueno, 1983; Rotter et al., 1993; Mogeda et al., 2002 and Hassan et al., 2003 and 2009).*** Also, fusarium mycotoxin inhibits cell division, RNA/ DNA synthesis and apoptosis **(*Rotter et al., 1996).*** Growth retardation and immune suppression are the major toxic effects induced by *Fusarium* ingestion in farm animals and suppression of the normal immune function and super induction of pro-inflammatory cytokines have been also suggested as supplementary tools for making a diagnosis as mentioned by ***(Widestrand et al., 2004 ; Kinser et al., 2004 and Hassan et al., 2004).*** This study, focused the highlight of the dangerous effects of fusarium and their mycotoxins pollution of animal feeds and water which allows a certain generalization as to the solution of problems regarding sheep breeding, which is an important contributor to the country's economy (especially at desert districts) in the form of meat, milk, wool and leather, with respect to the effects of environmental factors.

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The effects of CRP and PlGF expression on plaque stability in human carotid atherosclerosis

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Objective: This study aimed to investigate the effects of C-reactive protein (CRP) or placenta growth factor (PIGF) on atherosclerotic plaque stability. **Methods:** Fifty-five patients were recruited from among individuals who underwent carotid endarterectomy (CEA) in the Vascular Surgery department of the Fifth Hospital of Zhengzhou University from January 2008 to June 2009. The patients were divided into symptomatic and asymptomatic groups. Symptomatic patients were stratified according to the transient ischemic attack (TIA) frequency within six months: level I (1-2), level II (3-5), or level III (6 or more). CRP and PlGF expression were assayed and analyzed to determine whether they were associated with plaque stability. **Results:** No significant differences were found in CRP expression between the two groups, but PlGF expression in asymptomatic patients was lower in symptomatic patients. PlGF expression in asymptomatic patients was found to be positively related with TIA frequency, suggesting that lowering the PlGF level may represent an effective strategy to stabilize atherosclerotic plaques. [Life Science Journal 2010;7(3):58-63]. (ISSN: 1097-8135).

Keywords: carotid atherosclerosis, unstable plaque, placenta growth factor, C-reactive protein, transient ischemic attack

Introduction

Cerebral infarction (CI) is associated with some of the highest rates of disability and mortality. Even when promptly treated, most of the patients experience permanent disability. Cerebral infarction and atherosclerosis are closely related; atherosclerosis is a complex chronic inflammatory disease in which multiple factors and systems participate. Carotid atherosclerotic plaque rupture is considered the basis for symptomatic carotid artery stenosis, and may involve local inflammation and angiogenesis[1]. The accompanying platelet aggregation, thrombosis, internal haemorrhage, ulcer and defulvium are considered to be the principal pathophysiologic mechanisms leading to CI.

2 Materials and Methods

2.1 Study population and tissue sampling

A total of 55 patients were recruited for this study from among individuals who had Fifth Hospital of Zhengzhou University (China). All study protocols and procedures were approved by the local ethics committee of Zhengzhou University. The nature, purposes, possible benefits and risks of this study, other treatment options, rights and obligations were sufficiently explained to subjects individually. Written ǵCarotid atherosclerosis study informed consentô was obtained from each subject with no compulsion, improper pressure and temptation. Plaques were obtained by surgical excision. Resected tissues were rinsed within 10-20 minutes of removal, and then snap-frozen in liquid nitrogen and stored at -80℃ until analysis.

2.2 Study design

Resected carotid atherosclerotic plaques were divided into two groups according to preoperative clinical symptoms and examination results: Symptomatic group (SP) and Asymptomatic group (AP).

Inclusion criteria for SP included: at least one transient ischemic attack (TIA symptoms such as transient dizziness, amaurosis, limb asthenia and numbness, and salivation) within six months before surgery; and, manifestation of obvious local neurological dysfunction or single blindness,

regardless of whether the TIA signs were mild or permanent or if local carotid artery stenosis was >70%. This group was comprised of a total of 30 patients (Patient IDs: #1-#30), including 21 males and 9 females with an average age of 64.6 ± 12.7. Symptomatic patients were further stratified according to the TIA frequency experienced within six months: I level (1-2), II level (3-5), III level (6 or more).

Inclusion criteria for AP included: no history of TIA within six months before surgery; no clinical signs of nervous system dysfunction; and, presence of local carotid artery stenosis confirmed by CTA at >70%. This group was comprised of a total of 25 patients (Patient IDs: #31-#55), including 19 males and 6 females with an average age of 67.4 ± 7.2.

2.3 Sample preparation and protein analysis

Clear liquid between the upper oil phase and lower cloudy liquid phase was extracted from fully homogenized tissue, and enclosed in the 0.2ml EP and stored at -80℃ until use in analysis. We carried out protein quantification by use of the standard Bradford method using optical density $(OD)_{###}$ on a UV spectrophotometer (MODEL; MANUFACTURER, LOCATION) The CRP expression was assayed by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The OD_{450} value of each spot was determined, then a standard curve with absorbance OD value as ordinate (Y) and corresponding standard fluid concentration as abscissa (X) was generated. Western blotting was used to assay PlGF expression. Gel imaging and processing systems were used to perform scanning and image analysis to determine molecular weight and net value of target.

3 Results

3.1 Comparison of age and gender

χ^2 test with Ǔ=0.05 was used to compare the ages from among the AP and SP groups; the age range was not significantly different among the two (*P*=0.619). Studentôs *t*-test was used to compare the genders among the two, and again no significant difference was found (*P*=0.346).

3.2 Analysis of CRP concentration assayed by ELISA

In order to analyze total protein concentrations in samples from our patient cohort, a standard curve was generated first (Figure 1). From this, the standard curve equation was determined to be y = 0.0295x+ 0.0081，R^2 = 0.9867 and was applied to the evaluation of total protein concentration (εg/εl) of the protein extracts. The mean concentration from our 55 patients was 8.91 ± 4.92.

The standard curve was constructed according to the standard fluid absorbance (Table 2) to be used to determine the specific CRP protein concentrations in each sample. The equation formulated from this curve: y = 0.0036x + 0.0036，R^2 = 0.9935 was applied to the OD values of individual samples.

The resultant CRP protein concentrations were compared by studentôs *t*-test and variance analysis, with the level of significance set at Ǔ=0.05. The mean CRP protein concentration for the SP group was 21.44 ± 9.43εg/ml, and for the AP group was 25.27 ± 4.20εg / ml, (*P* =0.052). There were no significant differences (*P*>0.05) in CRP content of plaque tissues from the AP and SP groups.

3.4 PlGF expression assayed by Western blotting

The mean relative PlGF expression in the AP group was determined to be 2.02 ± 0.53, and that in the SP group was 0.58 ± 0.23. When the level of significance was set at Ǔ= 0.05, *P*=0.000, indicating that there was a significant difference in relative PlGF expression between the two groups. Specifically, the relative PlGF expression in AP was lower than that in SP (Figure 2).

3.5 Association of TIA frequency with instability of plaque

Stratification was performed in SP according to TIA frequency. Eight patients were characterized as level I, nine as level II, and thirteen as level III (Table 3).

We assessed the correlation between the grey level of symptomatic patients and TIA frequency by rank sum correlation test, R^2=0.915 when Ǔ=0.01.Correlation was statistically significant; thus, PlGF expression in plaques was highly correlated with TIA frequency in symptomatic patients.

4 Discussion

Our study found that there was a significant difference in PlGF expression in human carotid atherosclerotic plaque tissues from asymptomatic and symptomatic patients. PlGF

expression was positively correlated with TIA frequency, which suggests that PlGF could also be correlated with plaque instability. There were no differences found in the CRP expression in plaque from AP and SP. Moreover, CRP expression was not correlated with PlGF local expression in plaques. We inferred that CRP mainly reflects the condition of systemic inflammatory response, and has no significant effects on local plaque instability. PlGF is known to be involved in local angiogenesis of plaques, which can lead to plaque instability and the symptoms which characterize SP. Although the extent of carotid stenosis has been an important criterion for surgery, the carotid plaque instability is closely related with ischemic cerebrovascular disease. Therefore, the study of carotid plaque stability is of great significance to the prevention of ischemic stroke[2].

Carotid atherosclerosis is a part of general arteriosclerosis characterized by subintimal plate thickening of medium and large arteries. It may reduce or block blood flow. Together, the arterial wall thickening, hardening in the plaque site, lipid deposition and plaque necrosis characterize atherosclerosis. The subsequent events of plaque rupture and secondary thrombosis are the major pathophysiological basis for acute cardiovascular events.

CRP is a member of the pentamer protein family, which is secreted by liver epithelial cells in response to the presence of cytokine interleukin (IL)-6. Thus, as a sensitive inflammatory biomarker, CRP is considered a powerful predictor of cardiovascular events[3]. Previous studies have focused on the relationship between serum CRP and inflammation. We confirmed that CRP can also be detected in atherosclerotic plaques. Unfortunately, we did not find there were significant differences in CRP between SP and AP, which differed from our hypothesis, but was consistent with previous studiess[4]. The results of repeated assay comparisons of serum CRP between high and low risk people in were not consistent with our comparison between SP and AP. Different isoforms and post-translationally modified versions of CRP may explain this inconsistency[5].

PlGF is a kind of peptide growth factor that is known to bind with Flt-1/VEGFR-1[6], neural cilia protein -1 (NRP1) and neural cilia protein -2 (NRP-2) receptor, but specifically not with vascular endothelial growth factor (VEGFR-2)[7]. Studies into the molecular mechanisms underlying atherosclerosis have rarely involved PlGF. The As a result, the effects of PlGF on atherosclerotic plaque development remains unclear. Some studies have shown that PlGF, unlike VEGFR, is not necessary for embryonic development, despite the fact that it is highly expressed during pathological angiogenesis, such as in tumorigenesis or repair in response to trauma[8]. This finding suggested that PlGF may play a specific role in pathological angiogenesis.

Experience of a TIA event is a strong indicator of upcoming stroke. If the blood supply decreases for more than several minutes, the nerve cells in the ischemic region will die, leading to permanent neurologic impairment[10]. Recent studies have proven that in patients with carotid atherosclerosis, plaque rupture and thrombosis play vital roles in ischemic attack. The pathological angiogenesis may cause carotid atherosclerotic plaque instability, then rupture and thrombosis, which is relative to symptoms. Recent studies showed that angiogenesis may promote atherosclerotic lesions vulnerable to mechanical stress, leading to plaque internal hemorrhage[9]. Furthermore, our study found that TIA frequency was significantly related with PlGF expression levels in plaques. We inferred that the increased PlGF may lead to local plaque instability by encouraging pathological angiogenesis. Therefore, we may indirectly predict the outcome of stable patients by assaying serum PlGF level.

In addition, carotid atherosclerosis tends to occur in the initial segment of the carotid bifurcation, which suggests carotid atherosclerosis involves local factors. These factors that effectively increase intimal damage include the high shear stress of carotid blood flow and the mechanical damage of turbulence. When the sympathetic nervous system is activated by aggravating activities, agitation, cold or drugs, blood pressure becomes elevated, heart rate accelerated, and myocardial contraction enhanced; all leading to significant increase in mechanical stress suffered by plaques, including circumferential stress, shear stress, extrusion pressure which local artery spasm exerts on plaque, turbulence and so on. These stresses lead to plaque rupture. Circumferential stress is directly related to lumen diameter and intravascular pressure, while inversely proportional to vascular wall thickness. Recent studies on the shear stress of blood flow have revealed that complex shear stress is involved in endothelial cell activation.

Different mechanical movements in the blood stream have different effects on endothelial cells. Turbulence promotes cell proliferation and apoptosis, inflammatory reactions, absorption synthesis, and monocyte and lipid deposition. Thus, the possibility of plaque internal hemorrhage and rupture is supported. Increased apoptosis further encourages thrombosis and reduces the atherosclerotic plaque stability [11].

The commonly used treatments to stabilize atherosclerotic development include: 1) abstinence from cigarette smoking and wine intake; 2) changing diet style, such as eating more fish, and foods low in salt; 3) reducing abdominal fat; 4) performing more aerobic exercise; 5) inhibiting cholesterol synthesis by taking prescribed statins; 6) maintaining a normal hypoglycemic level; and, 7) supplementing the trace elements, such as vitamins and magnesium[12].

Carotid endarterectomy is a surgery routinely used to correct carotid stenosis and occlusions. It has become the gold standard for revascularization of the extracranial carotid occlusive disease. The CEA surgery may eliminate severe atherosclerosis stenosis, and also act as the second-level prevention for symptomatic patients[13]. In addition, CEA effectively reduces the stroke risk for asymptomatic patients[16]. With the development of science, carotid angioplasty and stenting (CAS) has provided a minimally invasive method for the treatment of extracranial carotid artery occlusive disease; trauma is minimal and patients receiving CAS recover quickly. Moreover, CAS facilitates treatment within the forbidden physical area known as the ósiphon segmentô, which CEA cannot reach. Maturity of CAS technology and equipment has led many physicians to consider CAS as having completely replaced CEA. However, a large-scale study showed that stent implantation was not equally beneficial for the treatment of carotid atherosclerosis plaques[14]. For patients who experience recurrent stroke, the optimal time window for surgery is two to five weeks after the condition is stabilized. Waiting more than 12 weeks may lead to further loss of brain cells[15].

5 Conclusions

The local PIGF level is able to better reflect plaque instability than CRP dose. Increased amounts of PlGF may promote plaque instability by encouraging pathological angiogenesis. Therefore, we may indirectly predict the outcome of stable patients by assaying serum PIGF level.

Table 1. Characteristics of experimental subjects

Group	Male (*n*)	Female (*n*)	Age (χ ± s)
SP	21	9	64.63 ± 12.73
AP	19	6	67.36 ± 7.18

Figure 1. Standard curve for protein quantification by Bradford method.

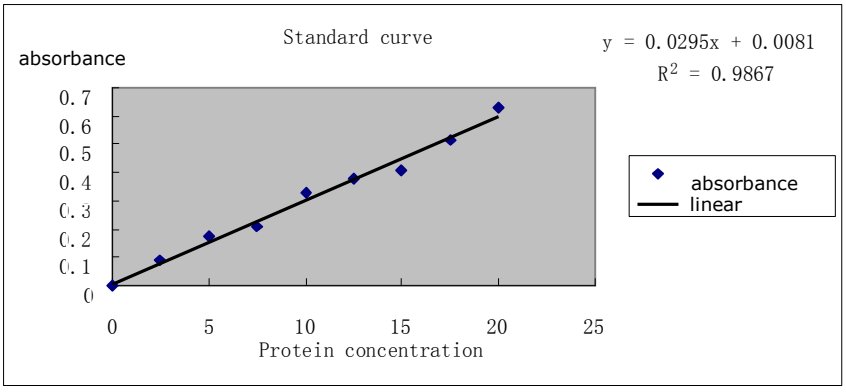

Table 2. ELISA standard curve

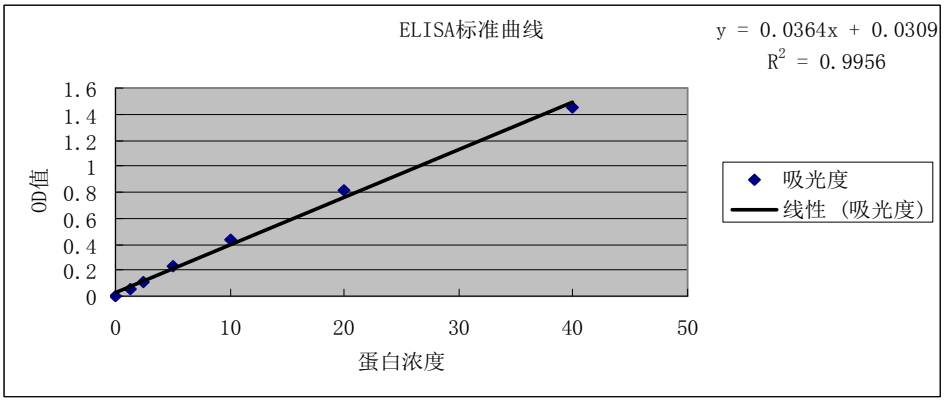

Figure 2. The expression image of standard protein ϐ-Actin and PlGF.

Note: 5, 18 and 27 were in the SP group; 39, 35 and 46 were in the AP group.

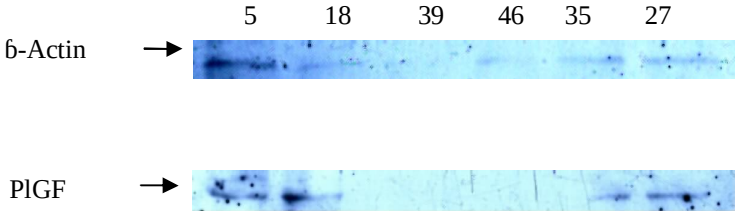

Table 3. TIA stratification and mean grey levels

TIA stratification	*n*	Mean grey level of SP group
Ⅰ level	8	1.45 ± 0.30
Ⅱ level	9	2.05 ± 0.45
ⅢIIIlevel	13	2.44 ± 0.35

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Effect Of Aflatoxin B1, Zearalenone And Ochratoxin A On Some Hormones Related To Fertility In Male Rats

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ABSTRACT

Three hundreds samples of feeds and sera of cattle and sheep (one hundred samples of each) were collected from farms at Minufiya, El-Behira and Assiute governorates in which animals (cattle and sheep) suffered from loss of weight gain, low productivity and disturbance in fertility. These samples were evaluated for mycotoxins contamination. Aflatoxins were detected in 30% of feed samples with the mean amount of 3.4 ± 0.1 ppm and ochratoxins in 20% with the mean values of 2.2 ± 0.02 ppm . Whereas, T-2 toxins and zearalenone were gained from 20% and 16% of samples with the mean levels of 36.0 ± 1.0 and 22 ± 0.3 ppm, respectively. But fuminosin B1 (FB1) toxin was found in 2% of samples at mean levels of 70 ± 0.01 ppm. The detection of mycotoxins in sera of diseased cattle and sheep showed that the most prevalent mycotoxins in cattle sera was aflatoxin B1 which detected in 40% of cases with the mean level of (5.4 ± 0.1), followed by ochratoxin A in 33% of cases with the mean level of (8.2 ± 0.1), T2 in 17% with the mean level of (26 ± 0.2) and zearalenon in (10%) with mean level of (19 ± 0.2). The lowest incidence was detected in cases 0f FB1 which obtained from 2% of cattle cases with the mean levels of (55 ± 0.6). Also , the pattern of incidence of mycotoxins in sheep sera were nearly similar to those in cattle with the exception that the FB1 not detected at all in sheep. The mycotoxins, aflatoxins, ochratoxins and zearalenone were given to male albino rats in the doses of 0.5, 1.0 and 2.5 ppm in feeds(respectively), for up to 6 months of age to investigate their effects on the growth rates and hormones regulating fertility (FSH, LH, Testosterone, T3 and T4). The results indicated the obvious adverse effects of mycotoxins on the secretion of these hormones and productivity of animals. The environmental pollutions particularly feed contamination was suggested to be the main source of the problem. Hence, regulatory measures must be undertaken to prevent such contaminants to reach the feed of animals. The significance of our results were fully discussed.

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Key Words: Mycotoxins, Aflatoxin, Ochratoxin A, Zearalenone, Fungi, Hormones, luteinizing H., follicle stimulating H. testosterone, thyroxin H.

Abbreviations: AF.: aflatoxin, T2: member of trichothecine toxins ,FSH follicle stimulating hormone, LH: luetinizing hormnone, T3& T4: tri-iodo-thyronine (total T3) and total thyroxin (total T4) hormones

1. INTRODUCTION

Up to date the progressive increase in world population require a parallel rise in the production of food. The majority of these food particularly that of animal origin may carry the dangerous factors for human and animal health. The fungal metabolites namely mycotoxins represent the most significant contaminants of food and feed **(Aly, 1993; Debey *et al*., 1995; Magnoli *et al*., 1999 and Hassan, 2003).** Various members of myoctoxins were detected in animal sera, feed and food and produced severe dangerous changes in active organs (**Hassan *et al*., 2004 and 2007and 2008 ; Hassan and Abd El-Dayem, 2004;).** The mycotoxins in feed consumed by animal and their serum cause disturbances in the hormonal profile related to fertility including follicle stimulating hormone (FSH), luetinizing hormone (LH), testosterone (TES.), thyroxin 3&4 (T3, T4) and can cause abnormal fetal development in farm animals which affect the normal function of reproductive organs and elsewhere the productivity of animals **(Xie *et al*., 1991; England *et al*., 1998; Tiemannand Vanselow, 2003; Hassan *et al*., 2003 and Ragheb and Srour, 2005).** Aflatoxins disturb the thymus gland functions which produced a significant reduction in feed intake and low body weight gain (**Fiorito *et al*., 1991 and Mocchogiani *et al*., 1998).**

However, the mycotoxins may be affected the semen quality which caused an increased in incidence of abnormal spermatozoa in animal and birds that administrated mycotoxins in diet **(Muthiah *et al*., 1997 and Yang et al., 2007 a & b),** who reported that treatment of mice with different doses of zearalenone caused increased numbers of abnormal spermatozoa and decreased amount of live spermatozoa with a significant reduction in body weight and relative epididymis weights. They also stated that after ingestion of contaminated cereals with fungi of fusarium and their toxins zearalenone, it may leads to animal fertility disturbances and other reproductive pathologies particularly suppressive effect on testosterone secretion. On the other hand, the mycotoxins reduced progesterone synthesis by inhibition the follicle stimulating hormone secretion (FSH) **(Tiemann and Vanselow, 2003).**

Therefore, the aim of the present work was to evaluate the effects of some mycotoxins on growth rate

and some the hormones related to fertility in male albino rats.

2. MATERIAL AND METHODS

2.1. MATERIAL:

2.1.1. Samples:

Serum and Feed samples: Three hundred samples of feeds and sera of cattle and sheep (one hundred samples of each) were collected from the farms at minufiya, El-Behira and Assiute governorates, in which animals (cattle and sheep) suffered from loss of weight gain, low productivity and disturbance in fertility. The feed samples were collected from store houses of these farms and the feeders of animals.

2.1.2. Strain for mycotoxins production: The mycotoxins were produced using toxigenic fungal strain as *A. flavus* (for aflatoxins), *A. ochraceus* (for ochratoxin A) and *Fusarium graminearum or F. roseum* (for zearalenone). These strains were isolated from animal feeds in laboratory of mycology of animal health research institute, Dokki.

2.1.3. Mycotoxins standard solution for TLC : Mycotoxins standard of Aflatoxins B_1, B_2, G_1, G_2, Ochratoxin A, Zearalenone , T2 and Fumonisin B1 were purchased from (Sigma Chemical Company, St. Louis U.S.A).

2.1.4. Laboratory animals:

One hundred apparently healthy male albino rats weighted (100-120 g) were housed under hygienic conventional conditions in stainless steel cages. Prior to experiment, rats fed on healthy basal diet free from any cause of disease. Drinking water was supplied in glass bottles, cleaned three times a week.

2.1.5. Biochemical reagents and kits for measurement the levels of hormones were purchased from Sigma chemical Company, USA.

2.2. Methods:

2.2.1. Detection of mycotoxins in serum and feeds:

Measurement of mycotoxins in serum and feeds was applied according to the fluorometeric method reported by **Hansen (1993).** The recommended amount of samples subjected for extraction of toxins by addition of methanol and water and passed over immunoaffinity column(each toxin have specific column).The obtained extract was measured by fluorometer or T.L.C.

2.2.2.Production of mycotoxins (aflatoxin, ochratoxin and zearalenone): The mycotoxins were produced using toxigenic fungal strain as recommended by **Smith (1997**) for aflatoxins**, by D'Mello *et al.*, (1997**) for zearalenone and by **Marquardt and Frohlich (1992)** for ochratoxins.

The isolates of *A. flavus*, *A. ochraceus* and *Fusarium graminearum or F. roseum*) which were isolated from feed samples and used for mycotoxins (aflatoxins, ochratoxin A and zearalenone) production, respectively. A flasks, each containing 100 gm of finely ground corn and 40-50 ml of distilled water was mixed and autoclaved at 121°C for one hour. The flask was shaken to prevent cooking of yellow corn. It was inoculated with corresponding fungus for required mycotoxins and incubated for 4 weeks at 25-28°C. In case of zearalenone production, the flasks were transferred to 8-10°C for additional 2 weeks. After end of incubation period , the corn was removed from flasks, dried, finely ground and 50 g of each was subjected to toxin extraction as recommended by **(Wyllie and Morehous, 1978 and Hansen (1993)).**

2.2.3. Experimental design:

A. The male albino rats were divided into 4 groups (25 of each). The first group kept as a control given healthy feed. Whereas, the second group given 0.5 ppm of aflatoxin in feed. However, the third group dosed with 1 ppm of ochratoxin in feed. The 4th group was treated by addition of 2.5 ppm of zearalenone in feed. The experimental work was continuoused up to 6 months.

B. Blood samples: During the experimental period (6 months), blood samples were collected every month from retro-orbital venous plexus **(Halperin *et al.*, 1951**) and serum was separated for determination the levels of LH, FSH, T3 and T4 hormones . At the beginning of 4th month of male age, blood samples were collected every 2 weeks till age of 6 month and serum samples were separated for estimation the level of testosterone hormone.

C. Body weight gain and growth rate were recorded every month from the beginning of the experiment till the end (1 to 6 month of age).

2.2.4. Determination of Luteinizing hormone (LH) and Follicular stimulating hormone (FSH) were performed according to the methods recommended by Santener *et al.* (1981).

2.2.5. Determination of serum testosterone hormone was performed according to the method described after Wilson and Foster (1992).

2.2.6. Estimation of serum total tri-odo-thyronine (total T3) and total thyroxine (total T4) hormones were performed as described by **Kornilvakayk *et al.* (1996).**

2.2.7. Statistical analysis: Data obtained were statistically analyzed using analysis of variance and comparing between groups were performed u-sing ANOVA test and Least Significant Difference (LSD) at $P < 0.05$ according to **Petrie and Waston (1999) and computerized using SPSS 11 (2002).**

3- RESULTS

In table 1, the mycotoxins were detected in feed and serum, samples collected from various farms of cattle and sheep at minufiya, El-Behira and Assiute governorates, in which animals were suffered from general low production, infertility and decreased growth rate. Aflatoxins were detected in 30% of the feed samples with the mean amount of 3.4 ± 0.1 ppm and ochratoxin in 20% with the mean level of 2.2 ± 0.2 ppm. However, it was revealed that the fusarium toxins were obtained at

lower rates (20% for T-2, 16% for zearalenone and 2% for fumonisin B1) with the mean amount of (36 ± 1.0 ppm, 22 ± 0.3 and 70 ± 0.00 ppm), respectively.

The sera of diseased cattle and sheep were examined for detection of mycotoxins and the results revealed that in case of cattle the most prevalent mycotoxins was aflatoxin B1 which detected in 40% of cases with the mean level of (5.4 ± 0.1), followed by ochratoxin A in 33% of cases with the mean level of (8.2 ± 0.1), T2 in 17% with the mean level of (26 ± 0.2) and zearalenone in (10%) with mean level of (19 ± 0.2). The lowest incidence was detected in cases of FB1 which obtained from 2% of cattle cases with the mean levels of (55 ± 0.6) (table, 2).

Also, the pattern of incidence of mycotoxins in sheep sera were nearly similar to those in cattle with the exception that the FB1 not detected at all in sheep(table 3).

However, the weight gain and growth rates were significantly decreased ($P<0.05$) due to administration of mycotoxins during the period of the experiment up to 6 month (Table, 4).

Moreover, the mycotoxins (aflatoxin, ochratoxins and zearalenone) reduced the LH and FSH in male rats which are related to regulations of normal productivity of animal (Table,5).

Data displayed in table (6) revealed a significant change in levels of testosterone of male rats which were administered mycotoxins in diet.

As shown in Tables (7&8) mycotoxins caused significant decrease ($P<0.05$) in T3, T4 and T3 / T4 ratio compared to controls.

DISCUSSION

The mycotoxins primarily enter the body systems of mammals by ingestion of contaminated food and feed stuffs and absorbed from alimentary tract. The principle point of entry of mycotoxins exposure for human and animals will be a direct consumption of contaminated food and feed **(D'Mello, 1997).**

The presences of mycotoxins in our study in feeds and sera of animals in Egypt was previously reviewed by **Hassan *et al.* (2002); Hassan (2003); Hassan *et al.* (2004) and Ragheb and Srour (2005),** who detected different mycotoxins in feeds and sera of animal including aflatoxin B1, zearalenone, T-2 and ochratoxin. They suggested that these toxins were responsible for the diseases of investigated animal cases and the rates of these toxins measures the severity of infection. The main effect of these toxins is the inhibition of protein synthesis throughout binding with DNA and RNA perhaps as a result of interference with nitrogen metabolism produced immunosuppresion and reduced antibody formation **(Zaghloul and Shehata, 1991; Hassan *et al.* 1997, 1998 and 2004).** Most of mycotoxins resulted in economic losses in animal wealth through their dangerous and carcinogenic effect in organs of animals particularly in liver, kidney and reduced productivity, reproductive insufficiency and male infertility**, (Wu *et al.*, 1991; Smith, 1997; Wang *et al.*, 2000 and Hassan *et al.*, 2004 and Ragheb and Srour, 2005).**

Therefore, the influence of mycotoxins on some hormones which are related to the regulation of normal productivity and fertility of male were investigated in present work. Similar to the obtained results of decreased weight gain, it was reported that the mycotoxins produced a variety of adverse health effects in farm animals, as inhibition of protein synthesis, reduction of feed intake which reflected in low weight gain **(Mocchegiani *et al.*, 1998; Hassan *et al.*, 2002; Hassan *et al.*, 2004 and 2008 and Ragheb and Srour, 2005).**

It is interesting to report here that the mycotoxins may be affected the semen quality which resulted increased incidence of abnormal spermatozoa in animal and birds administrated mycotoxins in diet (**Yang et al., 2007 a & b)**. Using of ANOVA test and comparison between groups that administrated mycotoxins resulted in detection a significant differences in levels of LH and FSH of male rats compared to controls. These results were previously reported by **Mitton *et al.* (1975); Smith (1982); Allen *et al.* (1983); Xie *et al.* (1991); England *et al.* (1998) and Hassan *et al.* (2004) and Ragheb and Srour (2005).**

The changes in levels of testosterone of male rats which were administered mycotoxins in diet could be attributed to low semen quality of breeders animal which affected by mycotoxins due to increased incidence of abnormal spermatozoa as a result of consumption of aflatoxin and zearalenone contaminated-diet (**Muthiah *et al.*, 1997and Yang et al., 2007 a & b)**. Also, the mycotoxins produced by several fungi in different food products or grains, cheese and meat, resulted in an drop in testosterone level by (66.6%) in male rats treated for 60 days **(Selmanoglu and Kockaya, 2004)**. The testis of treated rats with mycotoxins particularly patulin (a Penicillium and Aspergillus toxin) and zearalenone produced edema, fibrosis and local leydig cell hyperplasia in interstitial tissue and de-organization of semenifrous tubule epithelium and higher relative weight of seminal vesicle than those of control . These changes in testicular tissue resulted in insufficient of sperms production and reduced testosterone level lead to infertility of rats at different degrees and decrease the chance of normal reproductive activity **(Selmanoglu and Kockaya, 2004 and Yang et al., 2007 a & b)**..

The changes in thyroid functions hormones may be due to lymphoid cell infiltration and enlargement of interstitial tissue between follicle and degenerated colloid secretion in thyroid gland **(Selmanoglu and Kockaya, 2004).**

In the present work, the T4/T3 ratios showed lower results than the controls. This was detected in the hypo-function of thyroid gland, which was reflected in decreased feed intake, low body weigh gain and reduced productivity of animals ((**Fiorito *et al.*, 1991; Mocchogiani *et al.*, 1998 and Selmanoglu and Kockaya, 2004).** Also, many functional disturbances associated with hypothyroidism are due to a reduction in basal metabolic rate which resulted in decreased body weight gain without an associated change in appetite (**Botts *et al.*, 1991**). The hypothyroidism was reported to be responsible for abnormalities in reproduction of

breeding animal **(Wanda and Colin, 1998**). It could be results in reduction in testicular development and fertility of animals depending on the severity of the hormone deficiency **(Bell and Freeman, 1971).**

The public concern expressed about mycotoxins is not restricted to the effects of that mycotoxins contaminated food or feed on growth and health of animals and poultry but also about possible transmission of toxic residues in meat, milk, and eggs resulting in a potential hazard of human health **(Smith and Handerson, 1991).**

Conclusion

The results reported the significant influence of mycotoxins for some endocrine function of reproductive organs and thyroid gland which were reflected on the low productivity and high losses in animal wealth. The main source of these changes is attributed to the environmental pollution of food and feeds by fungi and their toxins. Therefore, every hygienic care must be undertaken during all steps of feed and food production and other factors related to the environment of animal to prevent such pollution. Hence the productivity of animal and human health become under control.

Table (1): Prevalence of mycotoxins in animal rations collected from farms in which animal suffered from loss of weight gain, low productivity and disturbance in fertility (ppm).

Mycotoxins	+ve samples	-ve samples	% of positive	Levels of mycotoxins (ppm)		
				Min	Max	Mean ± S.E.
Aflatoxin	15	35	30	1.5	1.5	3.4 ± 0.1
Ochratoxin A	10	40	20	1.0	3.2	2.2 ± 0.2
T2	15	40	20	2.0	50	36 ± 0.1
Zearalenone	8	42	16	10	30	22 ± 0.3
Fumonisin B1	1	49	2	70	70	70 ± 0.0

Table (2): Prevalence of mycotoxins in serum of diseased cattle.

Mycotoxins	+ve samples	-ve samples	% of positive	Levels of mycotoxins (ppm)		
				Min	Max	Mean ± S.E.
Aflatoxin B1	40	60	40	1.5	9.5	5.4 ± 0.1
Ochratoxin A	33	67	33	1.0	12.2	8.2 ± 0.1
T2	17	83	17	1.0	20	26 ± 0.2
Zearalenone	10	90	10	4	22	19 ± 0.2
Fumonisin B1	2	98	2	17	38	55 ± 0.6

Table (3): Prevalence of mycotoxins in serum of diseased sheep .

Mycotoxins	+ve samples	-ve samples	% of positive	Levels of mycotoxins (ppm)		
				Min	Max	Mean ± S.E.
Aflatoxin B1	35	65	35	2.5	11.5	8.6 ± 0.4
Ochratoxin A	25	75	25	3.0	14.1	10.5 ± 0.2
T2	4	96	4	0.8	17.0	12.1 ± 0.1
Zearalenone	8	92	8	2.2	19.0	8.9 ± 0.4
Fumonisin B1	0	100	0	0.0	0.0	0.0

Table (4): Influence of feeding a diet-containing mycotoxins (aflatoxin, ochratoxin and zearalenone) on weight gains (g) and relative weight ratio of male albino rats compared to controls (mean ± S.E.).

Treatment / Age (month)		Control (0.0)	Aflatoxin (0.5 ppm)	Ochratoxin (1.0 ppm)	Zearlaenone (2.5 ppm)	F-value
Weight gains (g)	1-2	1.04 ± 0.01^{A}	1.16 ± 0.02^{aB}	1.36 ± 0.018^{abC}	1.50 ± 0.027^{abc}	86.529#
	2-3	1.93 ± 0.04^{A}	1.78 ± 0.017^{aB}	1.43 ± 0.014^{abC}	1.93 ± 0.047^{bc}	50.799#
	3-4	1.43 ± 0.011^{A}	1.14 ± 0.015^{aB}	1.13 ± 0.022^{abC}	1.29 ± 0.045^{abc}	407.925#
	4-5	0.75 ± 0.024^{A}	0.04 ± 0.010^{aB}	0.02 ± 0.001^{aC}	0.51 ± 0.017^{abc}	517.167#
	5-6	0.57 ± 0.013^{A}	0.13 ± 0.011^{aB}	0.14 ± 0.013^{abC}	0.31 ± 0.017^{abc}	389.048#
Relative growth rates	1-2	46.03 ± 0.86^{A}	42.6 ± 1.67^{aB}	60.9 ± 1.24^{aC}	80.7 ± 1.31^{abc}	33.191#
	2-3	60.9 ± 1.05^{A}	52.2 ± 0.71^{aB}	41.4 ± 0.96^{abC}	59.5 ± 1.01^{bc}	89.339#
	3-4	4.04 ± 0.19^{A}	1.33 ± 0.07^{aB}	11.24 ± 0.52^{ab}	12.0 ± 0.25^{ab}	289.250#
	4-5	23.3 ± 0.46^{A}	1.32 ± 0.04^{aB}	0.63 ± 0.07^{ab}	14.5 ± 0.33^{ab}	447.945#
	5-6	0.79 ± 0.06^{A}	10.4 ± 0.71^{aB}	5.54 ± 0.37^{abC}	3.89 ± 0.19^{a}	92.997#

Significant at $P < 0.05$ using ANOVA test
Aa, Bb, Cc Significantly difference between two comparison groups in the same raw against capital litter at $P < 0.05$ using LSD.

Table (5): Influence of feeding a diet-containing mycotoxins (aflatoxin, ochratoxin and zearalenone) on serum leutinizing hormone levels (LH and FSH), (miu/ml) of male rats in comparison to controls values (mean ± S.E.).

Estimated Hormones	Treatment / Age (month)	Control (0.0)	Aflatoxin (0.5 ppm)	Ochratoxin (1.0 ppm)	Zearlaenone (2.5 ppm)	F-value
LH (miu/ml)	2	0.59 ± 0.02^{A}	0.59 ± 0.04^{B}	0.59 ± 0.04^{C}	0.39 ± 0.02^{abc}	7.278#
	3	0.69 ± 0.05^{A}	0.30 ± 0.01^{aB}	0.28 ± 0.02^{ab}	0.29 ± 0.02	38.210#
	4	0.90 ± 0.07^{A}	0.34 ± 0.02^{aB}	0.45 ± 0.04^{ab}	0.39 ± 0.02^{ab}	29.802#
	5	0.99 ± 0.05^{A}	0.19 ± 0.02^{aB}	0.15 ± 0.02^{ab}	0.20 ± 0.02^{ab}	128.403#
	6	0.91 ± 0.06^{A}	0.45 ± 0.05^{a}	0.60 ± 0.06^{aC}	0.55 ± 0.05^{ac}	10.761#
FSH (miu/ml)	2	6.27 ± 0.29^{A}	3.96 ± 0.50^{aB}	3.64 ± 0.32^{ab}	3.43 ± 0.31^{ab}	8.704#
	3	6.58 ± 0.49^{A}	4.02 ± 0.32^{aB}	3.22 ± 0.25^{ab}	3.23 ± 0.26^{ab}	6.452#
	4	6.06 ± 0.46^{A}	4.13 ± 0.19^{aB}	3.56 ± 0.30^{ab}	3.40 ± 0.32^{ab}	7.140#
	5	6.31 ± 0.46^{A}	4.67 ± 0.25^{aB}	3.55 ± 0.32^{ab}	3.47 ± 0.25^{ab}	6.511#
	6	6.91 ± 0.06^{A}	4.48 ± 0.26^{aB}	3.42 ± 0.29^{ab}	3.57 ± 0.25^{ab}	86.787#

Significant at $P < 0.05$ using ANOVA test
Aa, Bb, Cc Significantly difference between two comparison groups in the same raw against capital litter at $P < 0.05$ using LSD.

Table (6): Influence of feeding a diet-containing mycotoxins (aflatoxin, ochratoxin and zearalenone) on serum testosterone levels (ng/ml) of male rats compared to controls (mean ± S.E.).

Treatment / Age (month)	Control (0.0)	Aflatoxin (0.5 ppm)	Ochratoxin (1.0 ppm)	Zearlaenone (2.5 ppm)	F-value
4	7.46 ± 0.61^{A}	7.00 ± 0.64^{aB}	6.72 ± 0.55^{abC}	3.94 ± 0.31^{abc}	8.397#
4.5	15.16 ± 1.29^{A}	7.50 ± 0.64^{aB}	8.40 ± 0.77^{abC}	3.14 ± 0.39^{abc}	34.609#
5	13.98 ± 1.2^{A}	3.48 ± 0.46^{aB}	7.29 ± 0.79^{abC}	4.12 ± 0.55^{abc}	14.198#
5.5	15.12 ± 0.95^{A}	6.00 ± 0.64^{aB}	7.84 ± 0.46^{abC}	4.20 ± 0.44^{abc}	12.687#
6	12.22 ± 0.82^{A}	2.38 ± 0.46^{aB}	1.68 ± 0.23^{abC}	1.14 ± 0.18^{abc}	30.513#

Significant at $P < 0.05$ using ANOVA test
Aa, Bb, Cc Significant difference between two comparison groups in the same raw against capital litter at $P < 0.05$ using LSD.

Table (7): Influence of feeding a diet-containing mycotoxins (aflatoxin, ochratoxin and zearalenone) on serum triiodothyronine T3 (ng/ml) and thyroxine (T4) "ug/ml" of male albino rats compared to controls (mean ± S.E.).

	Treatment / Age (month)	Control (0.0)	Aflatoxin (0.5 ppm)	Ochratoxin (1.0 ppm)	Zearlaenone (2.5 ppm)	F-value
T3	2	224.0 ± 22.4^{A}	190.6 ± 22.5^{aB}	202.6 ± 11.4^{aC}	184.6 ± 6.52^{abc}	6.074#
	3	226.6 ± 19.3^{A}	184.6 ± 5.9^{aB}	189.4 ± 7.8^{aC}	164.6 ± 5.9^{abc}	7.048#
	4	219.6 ± 14.5^{A}	179.6 ± 6.52^{aB}	181.4 ± 7.87^{aC}	160.8 ± 5.82^{acb}	7.748#
	5	214.4 ± 4.89^{A}	140.8 ± 5.8^{aB}	143.6 ± 4.53^{aC}	136.6 ± 5.94^{abc}	9.584#
	6	231.4 ± 4.89^{A}	154.6 ± 6.52^{aB}	159.6 ± 6.52^{aC}	139.6 ± 8.87^{abc}	8.065#
T4	2	4.84 ± 0.27^{A}	3.84 ± 0.31^{a}	3.94 ± 0.30^{a}	3.64 ± 0.28^{a}	6.171#
	3	4.96 ± 0.43^{A}	4.04 ± 0.31^{a}	3.82 ± 0.27^{a}	4.06 ± 0.96^{a}	5.247#
	4	4.76 ± 0.50^{A}	4.48 ± 0.46^{a}	4.46 ± 0.49^{a}	4.36 ± 0.44^{a}	4.085#
	5	4.84 ± 0.56^{A}	4.14 ± 0.52^{a}	4.18 ± 0.46^{a}	4.22 ± 0.45^{a}	5.034#
	6	5.16 ± 0.50^{A}	4.34 ± 0.39^{a}	4.24 ± 0.31^{a}	4.36 ± 0.43^{a}	6.022#

Significant at $P < 0.05$ using ANOVA test
Aa, Bb, Cc Significant difference between two comparison groups in the same raw against capital litter at $P < 0.05$ using LSD.

Table (8) Influence of feeding a diet-containing mycotoxins (aflatoxin, ochratoxin and zearalenone) on serum T4/T3 ratio of male albino rats compared to controls (mean ± S.E.).

Treatment / Age (month)	Control (0.0)	Aflatoxin (0.5 ppm)	Ochratoxin (1.0 ppm)	Zearlaenone (2.5 ppm)	F-value
2	2.16 ± 0.26	2.01 ± 0.14	1.94 ± 0.19	1.97 ± 1.02	1.121
3	2.19 ± 0.30^{A}	2.19 ± 0.18^{B}	2.01 ± 0.21^{abC}	2.47 ± 1.74^{abc}	5.014#
4	2.16 ± 0.29^{A}	2.49 ± 0.16^{a}	2.45 ± 0.17^{a}	2.71 ± 2.11^{a}	6.123#
5	2.26 ± 0.25	2.94 ± 0.23^{aB}	2.91 ± 0.14^{a}C	3.08 ± 1.83^{a}bc	6.874#
6	2.23 ± 0.17^{A}	2.81 ± 0.26^{aB}	2.65 ± 0.11^{aC}	3.12 ± 2.08^{abc}	8.874#

Significant at P < 0.05 using ANOVA test
Aa, Bb, Cc Significantly difference between two comparison groups in the same raw against capital litter ay P < 0.05 using LSD.

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A General and Effective Two-Stage Approach for Region-Based Image Retrieval

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Abstract
Content-based image retrieval (CBIR) has received substantial attentions for the past decades. It is motivated by the rapid accumulation of large collections of digital images which, in turn, create the need for efficient retrieval schemes. Many research works further utilize regional features to obtain the semantics of images for better retrieval performance. In this paper, a two-stage retrieval strategy is presented to improve the performance of region-based image retrieval (RBIR). In this approach, an image is first segmented into a fixed number of rectangular regions. Then, each region is represented by its low-frequency discrete cosine transform (DCT) coefficients in the YUV color space. At the first stage of retrieval, the threshold-based pruning (TBP) serves as a filter to remove those candidates with widely distinct features. At the second stage, a more detailed feature comparison (DFC) is conducted over the remaining candidates. In the experimental system, users can represent their region of interest (ROI) by selecting different strategies, setting parameter values, and/or adjusting the weights of features as the search progresses. The experimental results show that both efficiency and accuracy can be improved by using the proposed two-stage approach. [Life Science Journal 2010;7(3):73-80]. (ISSN: 1097-8135).

Keywords: Content-based image retrieval; region-based image retrieval; threshold-based pruning; region of interest; discrete cosine transform.

1. Introduction

Digital content is becoming an important medium for image collection and exchange. Given the exploring market on digital photo and video cameras, the fast growing amount of image content further increases the need for image retrieval systems. Along this line, textual annotation of images is a simple and convenient way to express the image content. For example, modern search engines and their image search offspring have enabled significant progress in domains where visual content is tagged with text descriptions, but they only analyze metadata, not the images themselves, and thus are of limited use in many practical scenarios[1]. In practice, human annotation is not only subjective but also time-consuming. Besides, there is always a big gap in creating a mapping between words and visual features. To avoid many problems associated with annotation-based approaches, content-based image retrieval (CBIR) tends to index, sort, filter, and search images based on their visual content.

CBIR has received substantial attentions for the past decades. Some general reviews of CBIR literature can be found in[2-5]. Smeulder et al. reviewed more than 200 references in this field[2]. Datta et al. studied 120 of recent approaches[3]. Veltkamp et al. gave an overview of 43 content-based image retrieval systems[4]. Inoue reviewed the current research activities surrounding image access from the following aspects: information retrieval and organization technology, the infrastructure that enables large scale data processing, issues in human-system interaction, and the social issues[5]. These CBIR methods can be categorized into two major classes, namely, global methods and regional (or localized) methods[6]. Global methods exploit features characterizing the global view of an image while regional methods extract features from a region (or an object) of the image representing the visual content of it. Although global features can be extracted easily, in many cases, regional features contribute more meaningful image retrieval. To look at the regions (or objects) in the image, instead of looking at the image as a whole, is a way to obtain the semantic of an image, which is known as region-based image retrieval (RBIR)[7].

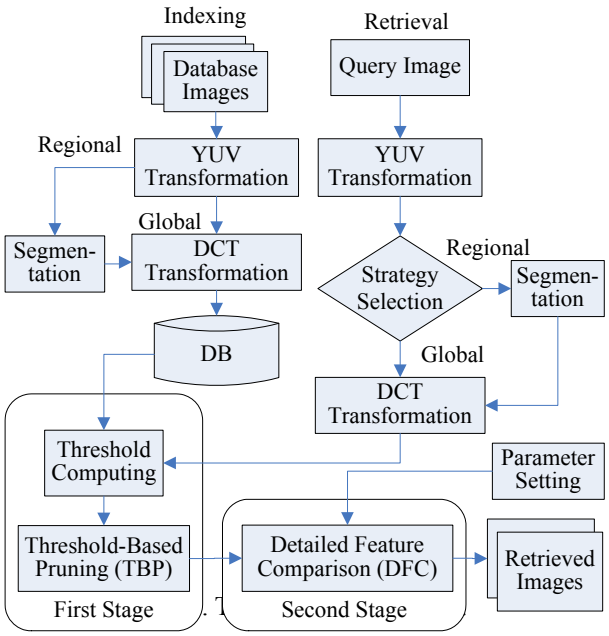
In RBIR, an image is required to be segmented into a number of regions with the aim of extracting the objects within it. However, there is no unsupervised segmentation algorithm that is always capable of segmenting an image into its constituent objects, especially when considering a database containing a collection of heterogeneous images. Therefore, an inaccurate segmentation may result in an inaccurate representation and hence in poor retrieval performance[8]. A number of RBIR systems has been presented[9-12]. In Blobworld[9], objects are recognized by segmenting the images into regions (blobs) that have roughly the same color and texture. The distance between two images is calculated as the distance between the blobs in terms of color and texture. The Netra system[10] segments images into region of homogeneous color and then uses the color, texture, shape and spatial properties for measuring similarity. The SIMPLIcity system[11] uses semantics classification methods, a wavelet-based approach for feature extraction, and integrated region matching (IRM) based upon image segmentation. The WALRUS system[12] uses wavelet-based retrieval of user-specified scenes. Each image is broken into overlapped sliding windows of varying sizes. The signature for each window is described by the lowest

frequency band of the Haar wavelet transform for the window. All these systems suffer the same problem that the segmentation may not have yielded regions close to the human perception of an object. The problem will become worse if a complex background is present in the image or no clear object is contained in the image. Besides, most automatic segmentation methods in RBIR include sliding-window search and region growing by pixel aggregation, region splitting and merging techniques, which are complex and computation intensive. Therefore, the size of the search space is sharply increased due to exhaustive generation of regions.

To cope with the above problems, this paper presents a two-stage retrieval strategy to model a RBIR framework. It includes three main parts: segmentation, regional feature extraction, and retrieval strategy selection. Segmentation and regional features are considered as black boxes, which means any segmentation algorithm and descriptor can be used within this framework. To reduce the problem of image segmentation, the images are first segmented into a fixed number of rectangular regions. Then, each region is described by its low-frequency discrete cosine transform (DCT) coefficients in the YUV color space. While conducting a query, the user can select the region of interest (ROI) in the query image to express his/her intentions. The similarity is evaluated by inspecting each region in the candidate images in turn, to find the best matching region with the query region. To let the system concentrate its effort on promising images, our two-stage retrieval strategy is applied. At the first stage of retrieval, the threshold-based pruning (TBP) serves as a filter to remove those candidates with widely distinct features. At the second stage, a more detailed feature comparison (DFC) is conducted over the remaining candidates.

A system framework is developed for realizing the proposed two-stage approach. It shows advantage over other RBIR systems in the following three aspects. First, during the retrieval process, color features and texture features are used in a two-stage way. To better combine these features, a friendly system user interface (UI) is provided to allow weight assignment for each individual feature. Second, the TBP mechanism employs an effective pruning algorithm to obtain potential candidate set, based on low-dimensional color and texture features of images. Last, a perception-dependent query strategy is proposed to support implicit relevance feedback. Instead of enforcing users to make explicit judgment on the results, the system UI simply lets the user select different strategies, adjust weights, and browse the results during the retrieval process. The experimental results show that the proposed framework is general, efficient and effective.

The remainder of this paper is organized as follows. The next section introduces the system framework for realizing the proposed two-stage approach. Section 3 discusses related issues about segmentation and region of interest. Section 4 illustrates the feature extraction process for an image or a region. The TBP method, which serves as the first stage of the retrieval, is shown in Sec. 5. Section 6 describes the second stage of the retrieval.



Section 7 explains how the performance is evaluated and Sec. 8 presents experimental results. Finally, some concluding remarks are drawn in Sec. 9.

2. The Proposed System Framework

To alleviate the burden of computation, the retrieval strategy is divided into two stages. The system framework that supports the proposed two-stage approach is illustrated in Figure 1. For the ease of extracting features, all images with different color-space are first converted into the YUV domain before they are forwarded in to our system. Then an image is equally divided into four rectangular regions and one additional central region of the same size. The DCT transform is performed over the Y, U, and V components for a whole image (global features) and five regions (regional features). In the indexing phase, the low-frequency DCT coefficients are all stored into the database.

When a query is submitted, the user can interact with the system by selecting different strategies and setting parameter values, such as ROI, threshold, standard deviation, and feature weights, etc. In the retrieval phase, at the first stage, the threshold-based pruning (TBP) serves as a filter to prune those images whose distances to the query image are beyond a distance threshold so that a smaller set of candidates is achieved. The threshold is obtained in advance by gathering the distances between the query image and database images. At the second stage, the detailed feature comparison (DFC) is performed on those resultant candidates passing through the first stage. This system also gets the user into the retrieval loop so that personalized results could be provided for the specific user.

3. Segmentation and Region of Interest

Image features for CBIR may be either "global" or "regional." A global feature is "coarse-grained," that is, it represents the image in its entirety, such as the overall color distribution. A regional feature is a finer-grained representation achieved through segmentation of the image into smaller regions. Both global and regional features offer advantages in processing and querying[13]. Global features offer advantages in terms of low computation complexity of feature extraction and pattern matching algorithms and can be used when queries deal with single entities. Regional features can be used to identify (or locate) objects within region of interest and to extract detailed information.

Object localization is an important task for the automatic understanding of images, which decides an object is present in an image or not, and even where exactly in the image the object is located[14]. There are a number of problems that generally hinder object localization, such as background noise, varying angles of view and object occlusion, and variations in image resolution and lighting conditions. We should note that though noise present in images interferes with image processing and interpretation, RBIR can still improve the performance because segmenting images into small regions would degrade the background effects.

To separate a region/object from the background, many different definitions of object localization exist in the literature. Typically, they differ in the form that the location of an object in the image is represented, e.g. by its center point, its contour, a bounding box, or by a pixel-wise segmentation. In the field of object localization with bounding boxes, sliding window approaches have been the method of choice for many years. Because the number of rectangles in an $n \times m$ image is of the order n^2m^2, one cannot check all possible regions exhaustively. Besides, the object is very hard to be identified because an image may contain several objects with varying positions and sizes, and one region may encompass different shades of the same hue, strong or weak textures, etc. In the approaches of the pixel-wise segmentation, the algorithms are complex and computation intensive, and the segmentation results are often not correct. For example, it is very hard to extract a region in complex natural scenes. The main difficulties arise from the intrinsic randomness of natural textures and the high-semblance between the objects and the background[15]. Instead of pursuing sophisticated segmentation methods, we segment each into a fixed 5-region layout (4 equal corner regions and an overlapping center region of the same size) as in the IBM TRECVID video retrieval system[16]. It has been shown that the segmentation is fast and thus suitable for a RBIR system with a large database.

In practice, even if a region is correctly detected, its visual appearance is not always specific to a single class of "objects" in a heterogeneous image database. Conversely some semantic "objects" can have very different visual appearance, such as "dog", "cloth", "car", etc. In other words, semantics and visual description do not always have a one-to-one correspondence. Besides, during the query process, it's hard to guess what region/object is the target of users, especially when multiple regions/objects are identified. The most straightforward solution of the problems is to let the user select a ROI while conducting a query. For example, if a query image contains various concepts: "train", "railroad", "sky", "trees", and "mountain", the user has to select the ROI that contains objects of interest. Our system UI allows the user to select any one of the five regions (upper left, upper right, lower left, lower right, and center) as the target region. As far as the target region is selected, the concept in the query image is more focused.

4. Feature Extraction

An image feature vector is a compact representation of how the image populates the feature space. In CBIR, the feature extraction will be invoked very frequently; therefore, too many items in a feature vector will make feature extraction and similarity evaluation become infeasible for an image retrieval application which requires instant response to a query. Generally in a CBIR system images are represented by three main features: color, texture, and shape. Each of these features has its own advantage to characterize a type of image content.

Some transform-based feature extraction techniques have been successfully applied to reduce the dimension of the vector in representing an image, such as wavelet, Walsh, Fourier, 2-D moment, DCT, and Karhunen–Loeve. Among these methods, the DCT is used in many compression and transmission areas, such as JPEG, MPEG and others. We use the low-frequency DCT coefficients as the color and texture features of an image. This is due to its strong "energy compaction" property: most of the signal information tends to be concentrated in a few low-frequency DCT coefficients. Some studies have shown the effectiveness of using the low-frequency DCT coefficients as feature vectors in CBIR[17-18].

Although the JPEG standard does not specify any particular color space for standard usage, our approach prefers the use of YUV color space in order to easily extract the features based on the color tones. All images with different color-space are first converted into the YUV domain before they are forwarded in to our system. The DCT is performed over the Y, U, and V components for a whole image (global features) and five regions (regional features). For an $N \times N$ image (or region) represented by pixel values $f(i, j)$, its DCT coefficients $C(u, v)$ can be defined as

$$C(u,v) = \frac{2}{N}\alpha(u)\alpha(v)\sum_{i=0}^{N-1}\sum_{j=0}^{N-1} f(i,j) \times \cos\left(\frac{(2i+1)u\pi}{2N}\right)\cos\left(\frac{(2j+1)v\pi}{2N}\right), \quad (1)$$

for $i, j, u, v = 0, 1, \ldots, N\text{-}1$, where $\alpha(w) = 1/\sqrt{2}$ if $w = 0$ and 1 otherwise. The feature vector for each image or region is further categorized into four groups: the DC coefficient (V_1) represents the average energy of the image and all the remaining AC coefficients contain three directional feature vectors: vertical (V_2), horizontal (V_3), and diagonal (V_4). For a 4×4 upper left corner of a DCT coefficient block, they can be defined as

$V_1 = [C_{00}]$,

$V_2 = [C_{01}, C_{02}, C_{03}, C_{12}, C_{13}]$,

$V_3 = [C_{10}, C_{20}, C_{30}, C_{21}, C_{31}]$, and

$V_4 = [C_{11}, C_{22}, C_{23}, C_{32}, C_{33}]$.

In our experiments, it can be observed that when taking into account directional textures (by setting combination weights to positive values through UI) the improvement in visual relevance of retrieved regions is usually noticeable.

5. The First Stage of Retrieval

The first stage of retrieval is to eliminate the obviously dissimilar candidates via a certain criterion. When an image sample is being queried, the candidates whose distances to the query image are beyond a distance threshold will be pruned at the first stage to narrow the candidate targets. Such a retrieval procedure is coined threshold-based pruning (TBP) in this paper. Note that TBP is conducted from the global view of images, not regional properties. In addition, psycho-perceptual studies have shown that the human brain perceives images largely based on their luminance value (i.e., Y component), and only secondarily based on their color information (i.e., U and V components). Therefore, both the U and V components can be of great help in removing those images with distinct color tones at the first stage of retrieval.

5.1 The Similarity Measurement

To exploit the energy preservation property of DCT, the sum of squared differences (*SSD*) is used to measure the distance of two images. Assume that $C_Q(u,v)$ and $C_X(u,v)$ represent the DCT coefficients of the query image Q and a candidate X in the Y component, respectively. Then the SSD_Y between Q and X under the upper left block of size $n \times n$ of the Y component can be defined as

$$SSD_Y(Q, X, n) = \sum_{u=0}^{n-1} \sum_{v=0}^{n-1} \left(C_Q(u,v) - C_X(u,v)\right)^2. \quad (2)$$

5.2 Threshold-Based Pruning

To realize the threshold-based pruning (TBP), we have to choose the suitable block size of DCT and the threshold T. A bigger candidate set after TBP means more probable to include good candidates at the cost of more computation. Decreasing the value of T reduces the amount of comparison at the sacrifice of excluding more potential candidates. Therefore, choosing the threshold T requires a certain amount of compromise.

In our work, the threshold values are derived by using the Chebyshev rule[19]. From this rule, we know that the percentage of observations contained within distances of k standard deviations around the mean must be at least $(1 - 1/k^2) \times 100\%$. For example, 75% is guaranteed within the distances of 2 standard deviations. Assume that T_n represents the threshold for the block size $n \times n$. To obtain the threshold T_n, the *SSD* between the query image and each candidate under the block size $n \times n$ is gathered. Thus, T_n can be derived by

$$T_n = \mu_n + k \times \sigma_n, \quad (3)$$

where μ_n and σ_n are the mean and standard deviation of *SSD*, respectively. Note that the Y, U, and V components are all used with a block size of 2×2 for the pruning criterion; therefore, μ_2 and σ_2 can be obtained by

$$\mu_{2(Y)} = \frac{1}{N_c} \sum_{i=1}^{N_c} SSD_Y(q, C_i, 2), \quad (4)$$

$$\mu_{2(U)} = \frac{1}{N_c} \sum_{i=1}^{N_c} SSD_U(q, C_i, 2), \quad (5)$$

$$\mu_{2(V)} = \frac{1}{N_c} \sum_{i=1}^{N_c} SSD_V(q, C_i, 2), \quad (6)$$

$$\sigma_2 = \frac{1}{N_c} \sum_{i=1}^{N_c} ((SSD_Y(q, C_i, 2) - \mu_{2(Y)})^2 + (SSD_U(q, C_i, 2) - \mu_{2(U)})^2 + (SSD_V(q, C_i, 2) - \mu_{2(V)})^2), \quad (7)$$

where N_c is the number of candidates in the database. The pruning process is denoted as TBP(YUV) for better understanding. The reason that Y, U, and V components are all used at TBP is to remove those images with widely distinct textures or color tones such that the return images will have a more consistent visual similarity with the query image whether they are uniform, textured or encompassing different shades of a given color.

To find out the best number of standard deviation, the threshold area provides an interface for the user to specify a threshold value in our system. Intuitively, setting the threshold value low can include more potential candidates. Since test results for CBIR systems are difficult to quantify objectively, a series of sample queries are conducted to access the mean performance. The finding shows that selecting any one of the thresholds did not cause any change in the top-10 list. Therefore, k=1 is the best choice because it provides 42.72% of data reduction rate, the highest one among four thresholds. The following experiments are all conducted based on the threshold under one standard deviation.

6. The Second Stage of Retrieval

In contrast to global match at the first stage, regional match at the second stage does a complete comparison between the query region and all regions in candidate images. To characterize images in a more detailed sense, a larger block size is involved at the second stage.

6.1 Detailed Feature Comparison

At the second stage, retrieval is performed by exhaustive comparison with query region, i.e., all regions in the remaining candidates that pass through the TBP filter are compared to the query region. The retrieval problem can be formulated as

$$dist(q, X) = \min_{x \in X} dist(q, x), \quad (8)$$

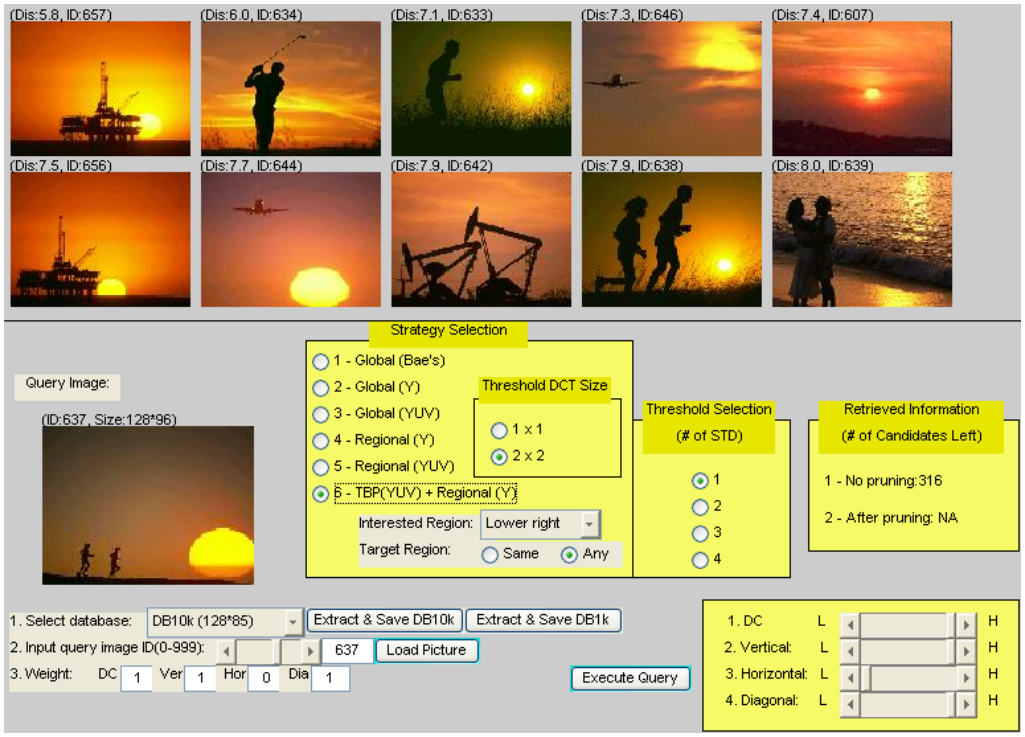


Figure 2. The main screen of our system, presenting results for a given query (image ID = 637)

where q is a query region and x is a region in a candidate image X. The distance between two regions is evaluated by detailed feature comparison (DFC). In DFC, only the Y components with a block size of 4x4 are used to calculate the distance between two regions. This process is denoted as DFC(Y) in comparison to TBP(YUV). The distance function in DFC(Y) is defined as

$$dist(q, X) = \min_{x \in X} dist(q, x)$$

$$= \min_{x \in X} SSD_Y(q, x, 4). \quad (9)$$

Note that the block size in DFC(Y) is larger than the block size in TBP(YUV), which means DFC(Y) is relatively detailed than TBP(YUV). Because the images with distinct color tones are removed at the first stage, only the Y component needs to be considered at the second stage for the purpose of efficiency.

6.2 Weighted Distance Measurement

In order to express users' semantic concepts with low-level features, the weights of these feature components have to be adjusted by users. This is important as users' interpretation varies with respect to different information needs and perceptual subjectivity. In addition, users tend to learn from the retrieval results to further refine their information priority. It is, therefore, useful to let users describe their perceptions of images by a set of weights, which also serves as a certain level of relevance feedback.

The distance measurements are defined in terms of weighted combinations of all features. Each weight associated to the individual feature is the user's personal interest to that feature. The distance function in DFC(Y) is modified to:

$$SSD_Y(q, x, V_i) = \sum_{C(u,v) \in V_i} (C_q(u,v) - C_x(u,v))^2, \quad (10)$$

$$SSD_Y(q, x) = \sum_{i=1 \text{ to } 4} w_i * SSD_Y(q, x, V_i), \text{ and} \quad (11)$$

$$dist(q, X) = \min_{x \in X} SSD_Y(q, x), \quad (12)$$

where q is a query region, x is a region in a candidate image X and w_i is the weight for V_i, indicating the significant level of the *i-th* feature. Note that V_1, V_2,V_3, and V_4 are the average grayness, vertical texture, horizontal texture, and diagonal texture, respectively. They are defined under the upper left block of size 4×4, as described in Sec. 4. The overall similarity is computed on the basis of the assigned weights, modeling what users see when they look at the query image.

7. Performance Evaluation

Generally, searches in CBIR are activities to decide which image is relevant for a certain purpose during the retrieval. For example, users usually search the database for images without assuming that the objects they are looking for are unique. Any objects described by the same information are good enough for a user's generic need. Therefore, some browsing is often involved in a CBIR system and only a short list of images is returned to the user.

Two commonly used performance measures, the *precision* rate and the *recall* rate, in textual information retrieval can be adapted for CBIR. *Precision* measures the ratio with which the relevant images are returned among

the best M matches; *recall* indicates the portion of relevant images that are returned among the best M matches. In practice, when the number of relevant images is greater than the size of the returned list, *recall* is meaningless as a measure of the retrieval quality. To overcome this problem, a measure called *efficacy* (η_M) is introduced[20]:

$$\eta_M = \begin{cases} n_r / N_r \text{ , if } N_r \le M \\ n_r / M \text{ , if } N_r > M \end{cases}, \quad (13)$$

where M is the total number of retrieved images, N_r is the total number of relevant images in the database, and n_r is the number of relevant images retrieved. If $N_r \le M$, η_M becomes the traditional *recall* of information retrieval; if $N_r > M$, η_M is indeed the *precision* of information retrieval. In our system, only the best 10 matches are returned, i.e., $M = 10$.

8. Experimental Results

For the experiments, we used an image database of 1,000 color images downloaded from the WBIIS image database[21]. It mainly consists of scenes of natural, animals, insects, building, people, and so on. No pre-processing was done on the images. Figure 2 shows the main screen of our RBIR system. The lower window on the screen gives the user possibility to edit some parameter values. For instance, the user can select the matching strategies, the position of ROI, the threshold DCT size, the number of standard deviations, or the weights for features. The top 10 matched images are displayed in the upper window, ranked in descending order of similarity to the query image (or region) from the left to the right and then from the top to the bottom. Figure 2 also presents a query example, showing the query results after the user loads a query image (ID = 637), selects the retrieval strategy and the ROI, adjusts the weights, sets other parameter values, and launches the query.

8.1 Evaluation of the YUV Color Space

One of the main aspects of color feature extraction is the choice of a color space. The RGB color space provides a useful starting point for representing color features of images. Alternative color spaces can be generated by transforming the RGB color space, such as CMYK (Cyan, Magenta, Yellow, and Black Key), CIE (Centre International d'Eclairage), YUV (Luminance and Chroma channels), etc. Each of the models is specified by a vector of values; each component of that vector being valid on a specified range. In our approach, the RGB images are first transformed to the YUV color space for the purpose of extracting the features based on the color tones more easily.

A sample image is purposely selected to illustrate the RGB and YUV color space, which contains three overlapped circles (see Figure 3). Each circle has one dominant color: red, green or blue. In this figure, for example, if we need to verify which part of a circle mainly contains a red color tone, it makes vain attempt to analyze the R component of the RGB image because most of the energy of the R component contributes to the luminance of the image; on the other hand, just like the philosophy lies in the orthogonal theorem, it is more effective to analyze the V component of the image, which eliminates the component that constitutes the luminance of the image. This vindicates the appropriateness of using the YUV color space in our approach.

8.2 Evaluation of Global Matching Strategies

Three global-based retrieval (or matching) strategies are examined in the following experiments: Global(Bae's)[22], Global(Y), and Global(YUV). Note that the letters in each parenthesis are the algorithm or the color components used in that strategy. The experimental results are summarized in Table 1, where efficacy is obtained by taking average over the retrieval results of query examples in each category. It clearly indicates that the efficacy of the Global(Bae's) approach is the lowest because it uses the RGB color space and does not sufficiently capture color information in the images. It can also be seen that Global(Y) is better than Global(Bae's) but still not good as Global(YUV), without the help of the U and V components. The finding suggests that Global(YUV) can serve as the criterion of TBP in our two-stage approach, removing the images with far distinct color or texture features from the candidates before getting into regional detailed feature comparison (DFC).

Table 1. The performance comparison of different global matching strategies.

Category ID	Category (# of Relevant Images)	Global match		
		Bae's	**Y**	**YUV**
1	Red apple (3)	50.0%	50.0%	100.0%
2	Green apple (3)	0.0%	50.0%	50.0%
3	Pumpkin (4)	22.2%	22.2%	77.8%
4	Deer (9)	50.0%	81.3%	75.0%
5	Horses(5)	31.3%	37.5%	43.8%
6	White owl (5)	30.0%	40.0%	75.0%
7	Eagle (10)	59.3%	61.9%	70.4%
8	Brown aninal (20)	75.0%	75.0%	95.0%
9	Sunset sky (83)	51.4%	54.3%	87.1%
10	Red rose (35)	86.0%	84.0%	96.0%
11	Cloudy sky (23)	75.0%	71.7%	96.7%
12	Mountain (90)	60.0%	62.2%	95.7%
13	Bear (14)	58.3%	60.0%	86.7%
	Average Efficacy	49.9%	57.7%	80.7%

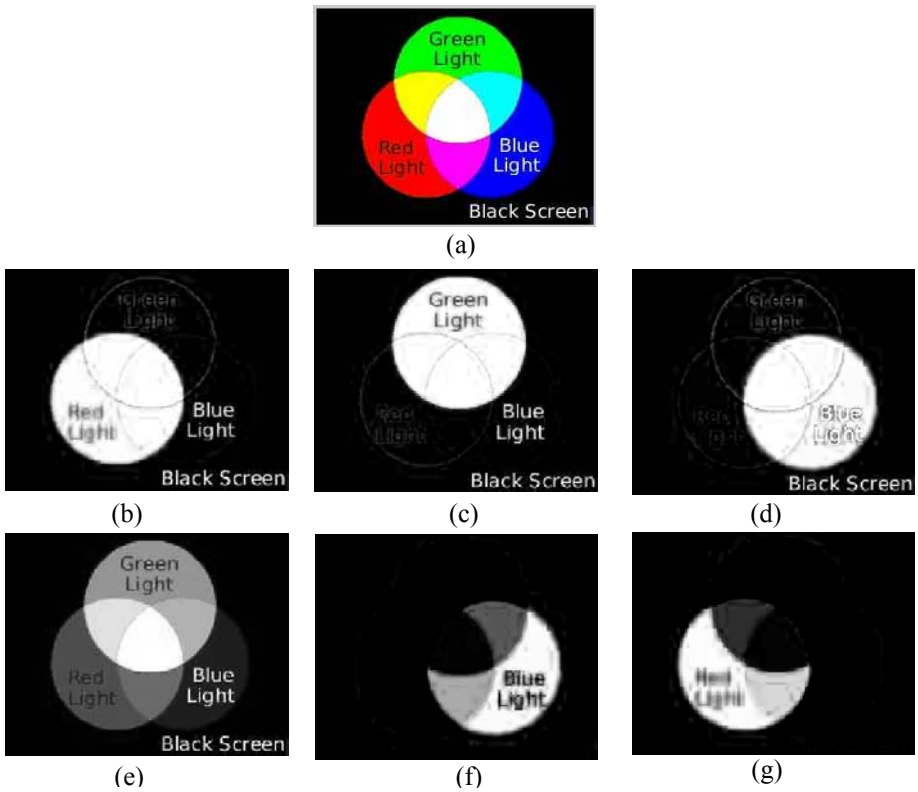


Figure 3. Illustration of RGB and YUV color spaces: (a) the sample image and the (b) R component, (c) G component, (d) B component, (e) Y component, (f) U component, and (g) V component of the image.

8.3 Evaluation of the Two-Stage Regional Matching Strategy

As there is no feature capable of covering all types of images, both global and regional features offer advantages in processing and querying. For those query images without clear objects, the user can select Global(YUV) to get the best results. If the user is only interested in a region that contains a target object, he/she can select regional strategies to conduct a more meaningful query.

The following experiments verified the proposed two-stage regional retrieval (or matching) strategy. We purposely choose ten queries with poor performance on the global match, compared with the same queries on the regional match. Table 2(a) shows the retrieval results for global match, where the queries use the whole image for feature construction without performing ROI selection. In the regional match, where the query is a region in an image, three regional matching strategies are studied and the results are summarized in Table 2(b): Regional(Y), Regional(YUV), and TBP(YUV)+Regional(Y). Note that the right most column shows the ROI, weights, and the number of relevant images. For convenience, the weights for four feature vectors are represented in the form of (w_1,w_2,w_3,w_4). We can see that Regional(Y) gives better performance than Global(YUV). It is reasonable because ROI would degrade the background effect even only the Y component is used. In comparison with Regional(Y), Regional(YUV) makes great improvement on the help of U and V components, raising efficacy from 41% to 77%. The improvement by the proposed approach, TBP(YUV)+Regional(Y), may not be very significant, but it decreases the running time because the total items of the feature vector in TBP(YUV)+Regional(Y) are less than those in Regional(YUV). It is also observed that instead of using the default weights (1,1,1,1), adjusting the weights will favor some queries. Users can adjust the weights through a series of experiments, with the goal of learning which features are most likely to contribute to the query.

9. Conclusions

In RBIR, computing similarity between two images is equivalent to region matching. Designing a RBIR system remains a challenging and open problem: automatic region segmentation is a hard task and its high complexity is generally a strong limitation due to the huge number of regions. In many cases, pixels which lie between segments or in high frequency areas of an image cannot be easily categorized as belonging to any particular segments. In this paper, we have simplified the problem in two aspects: 1) the number of representative regions for each image is only five, and 2) threshold-based pruning (TBP) reduces the matching to a smaller set of images. As can be seen in the experiments, segmenting images into small regions would degrade the background effects if the user is only interested in a portion of the query image. It is also shown that efficiency can be obtained while maintaining and sometimes improving the accuracy, by using TBP to prune those obviously unqualified candidates at earlier stages.

Table 2. The performance study of regional matching strategies.

	(a)Global	(b)Regional		
Image ID	Global (YUV)	Regional (Y)	Regional (YUV)	TBP (YUV) + Regional (Y)
604	3	9	9	lower left - (1,1,1,1) / 10
608	4	5	8	lower left - (1,1,0,1) / 8
609	2	6	9	center - (1,1,1,1) / 9
637	5	2	8	lower right - (1,1,0,1) / 9
645	3	4	8	lower left - (1,1,0,1) / 9
715	3	3	5	lower right - (1,1,0,1) / 6
716	4	3	9	center - (1,1,1,1) / 9
720	4	2	9	upper left - (1,0,1,0) / 9
727	1	4	5	center - (1,0,1,1) / 5
953	4	3	7	upper left - (1,1,1,1) / 7
Efficacy	33.0%	41.0%	77.0%	81.0%

In addition to fast segmentation and efficient matching, our approach also explores the user's presence in the retrieval, allowing the user to select the retrieval strategy, the point of interest and adjust the weights of features. With our friendly system UI, users can easily engage themselves in incremental query refinement and iterative retrieval by selecting different strategies and setting parameter values with the goal of exploring more interesting images. The experimental results show that our approach is general, efficient and effective. This evidence also suggests the potential application of the combination of fixed segmentation, flexible weight assignment, and two-stage retrieval for handling RBIR problems.

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Correlation of the Biological Traits of Cancers with Its Redox Status

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Abstract: Background The Redox status is a fundamental element for homeostasis, its deviation may be deeply involved in the pathogenesis of cancers. Here we investigate the deviation of representative redox pair of GSH/GSSG in cancers and its impacts on its biological traits. Materials and Methods The deviation of representative redox pair of GSH/GSSG in cancers was measured, including its changes in plasma of cancer patients and in tumor tissues, and its impacts on apoptosis, drug resistance of tumor cells and the tumor-neoangiogenesis. The state of GSH/GSSG in plasma and tumor tissues of cancer patients vs their control counterparts was examined by fluorometric analysis. The correlation of apoptotic factors of tumors with GSH/GSSG redox status were examened by immunohistochemical method in tissue microarray, the impact of GSH/GSSG redox status on proliferation of endothelial cells and on drug resistance of tumor cell were explored by MTT. Results The GSH/GSSG redox status in plasma of cancer patients deviated to pro-oxidative direction, while the GSH/GSSG redox status in cancer tissues deviated to reductive direction, which showed an opposite deviation vs that in plasma. The proliferation of endothelial cells stimulated by tumor-conditioned medium was totally reversed by GSH depletion. Depletion of intracellular GSH increased the adriamycin sensitivity in both MCF-7/ADM and MCF-7/S cells，and at the background of GSH depletion, the adriamycin exerted a significant reducing effect on intracellular GSH content in a dose-dependent manner. Discussions These results suggest that the GSH/GSSG redox status in cancer's plasma and cancer's tissues were differently deviated, which may be deeply involved in some unique traits of tumor cells, including the apoptosis, drug resistance of tumor cells and the tumor-neoangiogenesis. [Life Science Journal 2010;7(3):81-90]. (ISSN: 1097-8135).

Key words: Redox status; Cancer; Glutathione; Neoangiogenesis; Apoptosis; Drug resistance

Abbreviations

BSO	buthionine sulfoxine
CML-K562	chronic myelogenous leukemia K562 cell line
DEM	diethylmaleate
GSH	reduced glutathione
GSSG	oxidized glutathione
KCM	K562 tumor cell conditioned medium
MAPK	mitogen-activated protein kinase
MCF-7/ADM	adriamycin-resistant breast cancer cells
MCF-7/S	adriamycin sensitive breast cancer cells
MTT	3-(4,5-dimethyl-2- thiazolyl)-2,5-diphe tetrazolium bromide (thiazolyl blue)
ROS	reactive oxygen species

Introduction

Some unique traits of tumor cells give the cancer strong surviving advantage over the normal cells, such as the immortality, the anti-apoptosis, the capable of angiogenesis, even the drug resistance. These traits may involve a series of pathogenic mechanism, but the homeostasis of cancer must be different from normal cells in some aspects, and the altered homeostasis may be deeply involved in the occurrence and development of cancer, no matter as a attacking factor or as a consequences. Investigation of these alteration could possibly expose some intrinsic mechanism and clue some novel anti-tumor targets.

The Redox status, which describes the balance of oxidative-reductive biochemical reaction in the cells, is a fundamental element for homeostasis. Accumulating evidences have showed that the deviation of redox status might associated with the occurrence and development of tumors[1,2]. So the purpose of the present study was to investigate the potential involvement of the deviation of redox status in the regulation of some tumor's biological properties. Particularly in the influence of redox status on apoptosis of tumor cells, the tumors angiogenesis, and the drug resistance, which are very important factors for tumor's proliferation and anti-tumor therapy.

The GSH/GSSG redox pairs was selected as the marker of the redox status, the GSH is a essential intrinsic antioxidant protection mechanism and maintained at high concentration of millimolar level in tissues, which provide the intracellular milieu in a highly reductive state. The GSH/GSSG also functions in maintenance of tissue and plasma thiol-disulfide redox balance, the disulfide band, buffered by GSH/GSSG redox status, is extremely important for protein construction, the change-over of thiol-disulfide has been considered as a molecular switcher in regulation of gene expression, activities of enzymes, etc[3,4]. The GSH is also released from tissues,

and in the steady state, plasma GSH/GSSG redox values provide a means to distinguish between upstream oxidative events and downstream antioxidant events of oxidative stress.

So in the present study, the deviated degree of GSH/GSSG redox status in plasma of cancer patients compared with health subjects, and the deviation of GSH/GSSG redox status in cancer tissues compared with their counterpart paratumor tissues were examined, which would provide the background state of GSH/GSSG redox status in cancers. Then the anti-apoptosis, the capable of angiogenesis, and the drug resistance of cancer cells were investigated, and their relationship with the deviation of GSH/GSSG redox status were analyzed.

Materials and Methods

Materials

Adriamycin(ADM)-resistant human breast cancer cell line (MCF-7/ADM) and ADM-sensitive breast cancer cells(MCF-7/S), chronic myelogenous leukemia K562 cell line (CML-K562) were kindly gifted by Transplant and Immunity Laboratory of West China Hospital, Sichuan University, P.R.China. The umbilical cords for umbilical vein endothelial cell primary culture were obtained from health Caesarean birth in The Second West China Hospital, Sichuan University.

Assay of GSH/GSSG Redox Status in Plasma of Healthy Individuals and Cancer Patients

Subjects

120 healthy individuals (mean age 43.3 years old) from Sichuan University students and volunteers undergoing normal physical examination and 29 patients with cancer (mean age 43.5 years old) in digestive system from Department of Chemotherapy of West China Hospital, Sichuan University from 2003 to 2004 participated in the study. All participants gave their informed consent prior to their inclusion in the study and the study performed in accordance with the ethical standards of Declaration of Helsinki.

Measurement of GSH and GSSG in Homo Sapiens Plasma

The GSH and GSSG was measured by fluorescent-spectrophotometry modified from Hissin PJ[5,6].

Preparation of the GSH (Sigma) and GSSG (Sigma) standard curves

A series diluted GSH,GSSG mixed standard solution was prepared with the concentrations as 40、20、10、5、2.5nmol/ml of GSH and 20、10、5、2.5、1.25 nmol/ml of GSSG.

Table 1. Assay Protocols for GSH and GSSG

	standard GSH GSSG	&	NEM (2umol)	PBS	DTT (0.25umol)	PBS		’	PBS	OPA (1umol)
A’	50μL		50μL	0	0	150	A	100	1300	100μL
B’	50μL		0	50μL	0	150	B	100	1300	100μL
C’	50μL		0	0	50μL	150	C	100	1300	100μL

The GSH and GSSG standard curves was plotted with the GSH、GSSG concentrations as independent variable and measured fluorescent values as the function。

Measurement of plasma GSH、GSSG contents

Fasting blood samples were collected in the morning, the plasma was collected, deproteinized by addition of 1 volume of ice-cold 10% (w/v) metaphosphoric acid (MPA) and centrifuged at 10,000 g for 3 min. The following procedure was same with the preparation of GSH and GSSG standard curves，just replace the standard GSH & GSSG with 50μl deproteinized plasma, and the contents of GSH and GSSG in plasma were calculated from the standard curves.

Measurement of GSH/GSSG，the Apoptosis-related Factors in Tumor Tissues and Paratumor Tissues and Their Relationship Analysis

Collection of tissue specimens and preparation of tissue homogenates

Tumor tissue and corresponding paratumor tissue were obtained from surgery excised specimens in 42 patients with digestive system cancer. The tumor type included esophageal cancer (13 cases), gastric cancer (14 cases) and colon cancer (15 cases). Corresponding paratumor tissues were taken from the resected specimens where it appeared healthy under light microscope, about 5-10 cm distant from the tumors tissue. Parts of tissue specimens were immediately frozen in liquid nitrogen after excision and kept at －70°C refrigerator until analysis for GSH/GSSG.

0.05g tissue specimen was homogenazed with 0.75ml sodium phosphate-EDTA (pH 8.0) and 0.2 ml 25% MPA at ice-water bath, then centrifuged at 4000rpm for 15 min, the supernatant was collected for assay of tissues GSH and GSSG contents.

Determination of GSH and GSSG in tissues

0.2ml tissue homogenates supernatant were diluted for 2000 times with sodium phosphate-EDTA, the measurement for tissue’s GSH/GSSG is similar to which in plasma.

Determination of apoptosis-related factors

Parts of the cancer and paratumor tissues were paraffin wax embedded, and arranged in a tissue chip (tissue microarray) for immunohistochemical staining.

The tissue chip was prepared by cybrdi biotech Ltd (China), the first antibody was mouse polyclonal antibodies (Wuhan Boster biotech, China) for Bcl-2, Bax, NF-κB and rabbit polyclonal antibody (Wuhan Boster biotech, China) for caspase-9, and the second antibodies were from sheep serum. The routine immunohistochemical procedure was carried out with the negative control by PBS for replacement of the first antibodies. The immunohistochemical images were

analyzed by software of IMAGE-PRO plus4.1. Then the correlations between the expression of apoptosis-related factors and the indexes of redox status were analyzed impacts of GSH/GSSG redox status deviation on the proliferation of endothelial cells potentiated by tumor cell-conditioned medium

This part of the study is aimed to investigate whether the deviation of GSH/GSSG redox status correlates with the proliferation of endothelial cells potentiated by tumor cells, and hereby to explore the anti-tumor possibility through regulation of GSH/GSSG redox status.

Preparation of tumor-conditioned medium and Cell culture

CML-K562 cell (Chronic myelogenous leukemia line K562) were seeded at 2.5 ×105/ml in RPMI-1640 medium (RPMI-1640) supplemented with newborn calf serum (Beijing Baolanbo Biol-Tech)) for 24 hours, the post-culture medium were harvested and centrifuged at 1500rpm for 20 min to remove cell debris, and the collected supernatant was assigned as K562 cells conditioned medium (tumor-conditioned medium KCM) and stored at - 20 ℃ until use.

Endothelial cells were isolated from Homo sapiens umbilical cord and cultured in medium-199 (GIBCO) supplemented with 20% newborn calf serum. The cell identifying test with antibody to Factor Ⅷ-related antigen showed that more than 95% of cells were positively stained, and the cells grew to a typical cobblestone morphology when confluent..

Experiment groups

The endothelial cells in exponential phase were harvested and then cultured for 24 h with RPMI-1640 medium contained 4% newborn calf serum to reach synchronization. The synchronized cells were divided into three groups: (a) RPMI medium without any more treatment as control; (b) various concentrations of KCM (v:v = 0%, 20%, 40% respectively) were added into the culture; (c) various concentrations of KCM (same with b) and 1.5mmol/L BSO (Sigma) were added into the culture. All cells continued culture for another 48 h.

Evaluation of the viability of endothelial cells in KCM and their intracellular GSH/GSSG redox status

After 48 h treating, one bolus of endothelial cells was used for assay of cells viability by MTT test (Sigma). Another bolus of endothelial cells were treated by trypan blue for viable cell counts, and other cells were lysed by repeated freeze (at -70℃) and thaw (four times) and centrifuged. The supernatants were used for GSH and GSSG assay.

Impact of Depletion of Intracellular GSH Content on Drug-resistance of Tumor Cells

First, the human breast cancer resistant line cells to ADM (Pharmacia ＆Upjohn Company, Italy) (MCF-7/ ADM) and sensitive cell lines (MCF-7/S)were treated by different concentration adriamycin (ADM) respectively, cell viability were measured by MTT, and the IC50(the drug dose for 50% survival rate) and multiple coefficient for the drug-resistibility between the two kinds of cells were calculated.

The MCF-7/ADM cells were cultured to exponential phase, then transferred to serum-free medium for 24 h to reach synchronization, the synchronized cells were divided into four groups: (a) MCF-7/ADM cells were cultured in RPMI-1640 medium as control; (b) MCF-7/ADM cells were treated with different concentrations of ADM; (c) MCF-7/ADM cells were treated with 0.1 μmol/L DEM for 3 h, then the medium was renewed with RPMI-1640 medium ; (d) MCF-7/ADM cells were pretreated with 0.1 μmol/L DEM for 3 h, medium was then renewed with RPMI-1640 medium and ADM was added with different concentrations as (b). All cells were cultured for further 24 h.

The survival rate before and after DEM was measured by MTT, and different concentration ADM was then added for observing the changes of drug-resistance after DEM. The GSH concentration in MCF-7/ADM cells was examined simultaneously by fluorescent-spectrophotometry，the correlation between the changes of resistance to ADM and the intracellular GSH concentration was analyzed.

The MCF-7/S cells were also treated by DEM for 3 hours，and the changes of sensitivity to ADM and intracellular GSH content were examined as in MCF-7/ADM cells.

The diethylmaleate (DEM, Beijing Baolanbo Biol-Tech, China) is a pro-oxidant、glutathione-depleting agent, which can decrease intracellular GSH level in a dose-dependant manner.

Statistical Analysis

Data are expressed as means ± standard error of means (SEM). Data were analyzed using one-way analysis of variance (ANOVA), t-test and linear correlation analysis by SPSS 12.0 software, and the difference was considered significant when $P< 0.05$.

Results

GSH/GSSG Redox Status in Cancers Plasma and Tissues Deviated Differently from Healthy Plasma and Paratumor Tissues.

The results in table 2 displayed that the GSH content and GSH/GSSG ratio in plasma of cancer's patients declined compared with healthy individuals ($P<0.05$), which indicated a deviation of GSH/GSSG redox status to pro-oxidative direction. The cancers tissues, however, expressed an opposite deviation of GSH/GSSG redox status to reductive direction, the GSH content and GSH/GSSG ratio in cancer's tissues were significantly higher than their counterparts of paratumor tissues ($P<0.01$).

The redox status in plasma usually represented a general condition of cancer's patients, while the redox status in cancer tissues may more directly reflect the actual state in cancer's itself. The direction-different deviation of GSH/GSSG redox status in the two sources of cancer samples could imply some underlain intrinsic mechanisms involved in the tumor's biological properties, which will be further referred in discussion.

The Different Expression of Apoptosis-related Factors in Cancer and Paracancer Tissues and Their Correlation with the Deviation of GSH/GSSG Redox Status

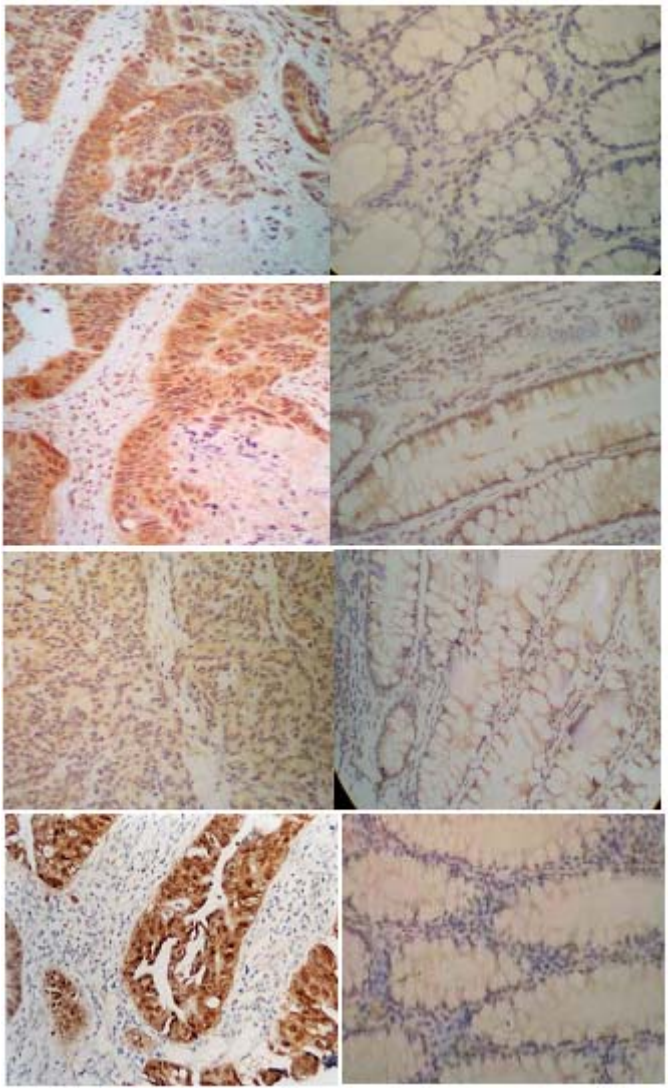

Fig 1. The Immunohistochemical Staining of Bcl-2、Bax、caspase-9 and NF-κB in Sequence from Top to Bottom, the left column is cancer tissues and the right column is corresponding paracancers tissues. The images in left column showed much stronger positive staining.

Owing to the positive- proliferating effects of NF-κB activation on cell, the increased Bcl-2 expression and the reduction-deviated redox status in carcinoma tissues, which furnishes the cancer anti-apoptotic trait, and the malignant cells showed a great proliferation capability. Results from correlations analysis with redox status showed a obvious correlations between the expression of Bcl-2 and the GSH/GSSG ratios (Fig. 2).

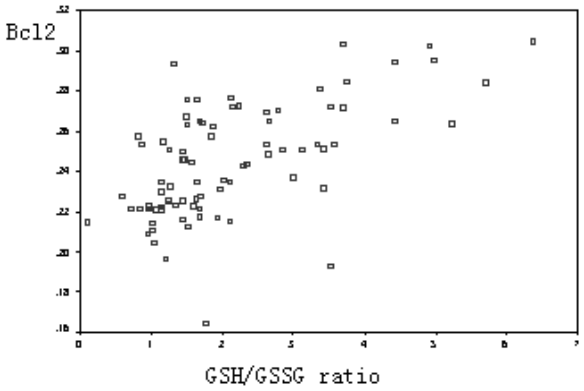

Fig 2 Correlation between Expression of Bcl-2 and the GSH/GSSG Ratio in Cancers and Corresponding Paracancers Tissues ($r=0.603$. $P<0.01$)

Table 2. GSH/GSSG Redox Status in Plasma and Tissues of Healthy Subjects and Cancer Patients

Samples	subjects	n	GSH(nmol/ml)	GSSG(nmol/ml)	GSH/GSSG ratio
Plasma	Cancer patients	29	6.01±0.65*	0.74±0.11	8.39±1.81*
	Healthy individuals	120	7.47±1.50	0.66±0.05	11.44±2.45
Tissues	Cancer tissues	42	584.88±207.93#	62.91±94.94	9.78±1.38#
	Paratumor tissues	42	322.49±88.81	64.99±114.80	5.51±0.66

* P<0.05 vs healthy individuals, # P<0.01 vs corresponding paratumor tissues

Table 3. The Expression of Bcl-2,Bax, Caspase-9 and Nuclear Positive Rate of NF-κB in Cancers and the Corresponding Paratumor Tissues（$\bar{x}$ ±s）

Groups	N	Bcl-2	Bax	Bcl-2/ Bax	Caspase-9	nuclear positive rate of NF-κB (%)
cancer	42	0.264±0.020*	0.275±0.013*	0.963±0.080*	0.262±0.017*	21.63±3.16*
paratumor	42	0.225±0.019	0.263±0.020	0.862±0.098	0.246±0.019	4.13±0.95

*P<0.01 vs the corresponding paratumor tissues

The expression of Bcl-2 as one of suppression factors of apoptosis, the Bcl-2/Bax ratios and the nuclear positive rate of NF-κB obviously increased in cancer tissues compared with their corresponding paratumor tissues (Fig.1).

These results suggested that the increased antioxidative capability in cancer tissues is one of the important reasons for their great anti-apoptosis and proliferation capability. The expression of Bax and caspase-9, pro-apoptotic factors, increased too. The oxidative stress in cancer tissues is probably contributed to this increase.

Depletion of Intracellular GSH Reverses the Proliferating Activity of Endothelial Cells Inspired by Tumor-conditioned Medium

A promising tumor-treating remedy has been designed to antagonize the neoangiogenesis of tumor through inhibiting tumor-secreted proliferating factors, such as the HIFα、VEGF、FGF、PDGF etc, and their receptors, which were thought to be the initiating and developing engine in angiogenesis of tumor microvasculature. But how is the response of endothelial cells, which were not the malignant cells, to these proliferating factors? as the essential constituent of the tumor microvasculature, endothelial cell may also be deeply involved in the regulation of this neoangiogenesis, some biological properties of endothelial cells in this proliferating process under the influence of tumor tissues could be hence a potential novel target in anti-neoangiogenesis for tumor therapy.

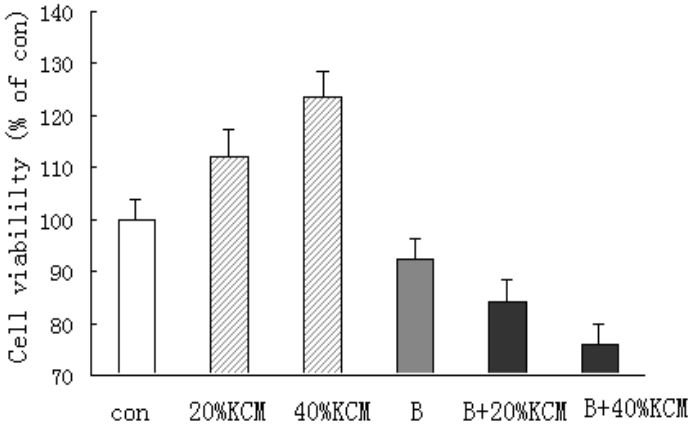

Figure 3 Impacts of KCM and BSO on Proliferating Activity of Endothelial Cells
KCM : tumor conditioned medium;
BSO: buthionine sulfoxine, inhibitor of GSH synthesis

The result in figure 3 illustrated the inspiring effects of tumor conditioned medium KCM on endothelial proliferation, and the reversing effects on this inspiring effects by BSO treatment, which is a specific inhibitor of de novo GSH synthesis. The proliferation of endothelial cells was inspired by KCM in a dose-dependent manner without BSO treatment, but adding the BSO into the culture system, which decreased the endothelial intracellular GSH content, the situation was totally turned over, the promoting effect on proliferation of endothelial cells by KCM was reversed even in a negative dose-dependent manner, the endothelial viability showed a downhill trend along with the increasing concentration of KCM in BSO treating groups(Figure 4).

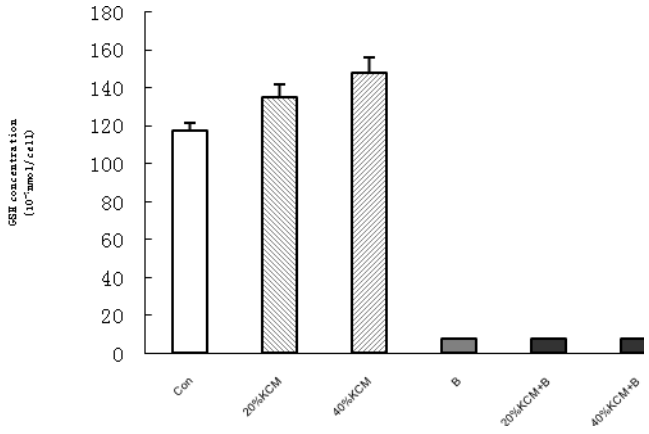

Figure 4 Impacts of KCM and BSO on Intracellular GSH Concentration of Endothelial Cells
(P<0.01 without BSO vs with BSO，and between different KCM groups)

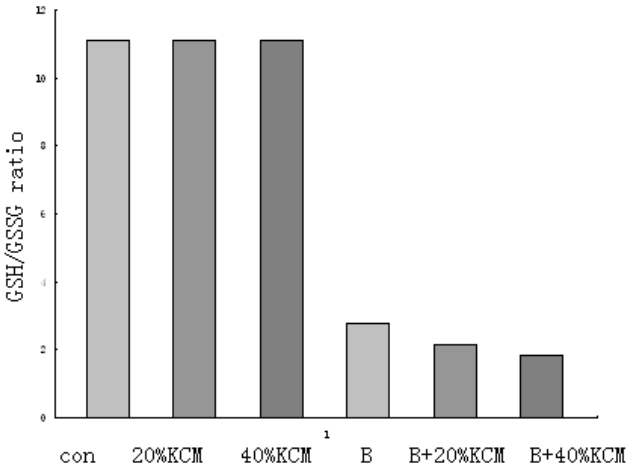

Figure 5 Impacts of KCM and BSO on GSH/GSSG ratio of Endothelial Cells
(P<0.01 without BSO vs with BSO，and between different KCM groups)

Figure 4 and 5 illustrated the impacts of KCM on GSH/GSSG redox status of endothelial cells with and without BSO. Figure 6 analyzed the correlation between the proliferating activity of endothelial cells and its intracellular GSH/GSSG redox status with the KCM and BSO treatment.

The result from figure 4 showed that the intracellular GSH concentration in endothelial cells was enhanced by KCM treatment in a dose-dependent manner. After BSO treatment, however, the GSH concentration greatly declined, and no enhancing effect by KCM was observed along with the increased KCM concentration.

The interesting result in figure 5 was that if we take the GSH/GSSG ratio, but not the GSH content as the parameter, the KCM didn't change the GSH/GSSG ratio, which means the GSH/GSSG redox status of endothelial cells was maintained under the KCM stimulation, different from the result in figure 4, where the GSH content was enhanced by KCM. This fact however, may suggest a concert with the results in the above part of GSH/GSSG redox status in tissues and plasma of cancers patients, where a coexist of increased GSH content in cancer tissue and oxidative stress in plasma may suggest that the tumor could maintain a balanced redox status via increased GSH content in face of oxidative stress. Similar with this, the endothelial cells was also maintained a balanced redox status during the stimulation of tumor conditioned medium KCM.

But if we depleting the intracellular GSH in BSO groups, the KCM was exhibited a pro-oxidant effect on GSH/GSSG redox status in endothelial cells, the GSH/GSSG ratio in endothelial cells displayed a potential dose-dependent decline with the increased KCM concentration(figure 5).

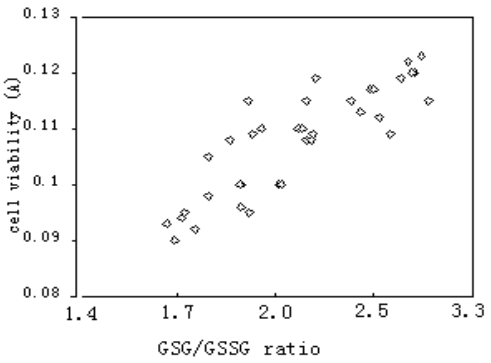

Figure 6 The Correlation of Proliferating Activity of Endothelial Cells with Its Intracellular GSH/GSSG Ratio （r＝0.865，P<0.01）

Analyzing the correlation of proliferating activity of endothelial cells with its intracellular GSH/GSSG redox status displayed a close relationship between the two properties, the coefficient was r＝0.865 (P<0.01) as in Figure 6.

Depletion of Intracellular GSH Dose-dependently Reverses the Drug Resistance of Cancer Cells

Another important characteristics of cancer cells, which may involve the intracellular redox status, was the drug resistance. In the fourth part of the study, we measured the intracellular GSH contents in cancer cells of human breast cancer cell line MCF-7 with adriamycin resistant(MCF-7/ADM) or adriamycin sensitive(MCF-7/S), the MCF-7/ADM cells showed a higher GSH concentration (10.70±1.58nmol /106cells) than MCF-7/S have (6.29±0.67nmol/106cells). The IC50 (the drug dose for 50% survival rate) for adriamycin in MCF-7/ADM cells was much higher than the IC50 value in MCF-7/S cells.

Pretreatment of MCF-7/ADM cells with DEM, which is a intracellular GSH depleting agent, the IC50 value for adriamycin in MCF-7/ADM cells downed from 1075.72±30.20mg/L to 314.11±30.17 mg/L as shown in table 3.

Table 3 The Intracellular GSH (nmol /106cells) Content and the IC50(mg/L)for ADM in Breast Cancer MCF-7 Cells

Human breast cancer cell line	MCF-7/S	MCF-7/ADM	MCF-7/ADM treating with 0.1μmol/L DEM for 3 h
IC50（mg/L）for ADM	28.10±0.98	1075.72±30.20	314.11±30.17
Intracellular GSH (nmol /106cells)	6.29±0.67	10.70±1.58	Dose-dependent declined along with the increased ADM concentration (see figure 5)

The figure 7 showed that the treatment with DEM or ADM alone just had a mild to moderate effects on GSH decline. After DEM pretreatment however, the ADM exerted a significant dose-dependent reducing impact on intracellular GSH content, which was even much stronger than the simple added effects of DEM+ADM. The cancer cell viability exposed a close relationship with the intracellular GSH content as shown in figure 8.

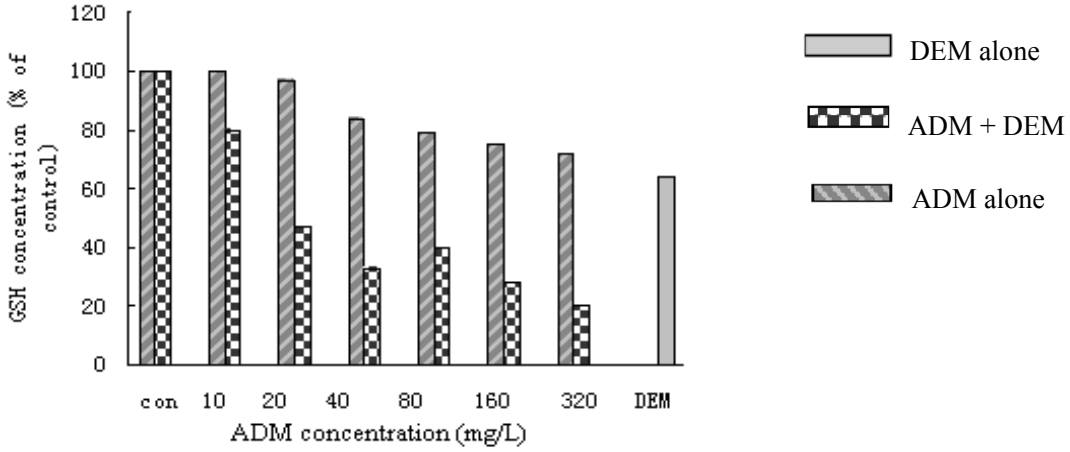

Figure 7. Changes of GSH Concentration in MCF-7/ADM Cells with or without DEM Pretreatment. MCF-7/ADM cells were pretreated with 0.1umol/L DEM for 3 hr. Medium was renewed and ADM was added at increasing concentrations for further 24 hr incubation , GSH concentration was then assayed. The GSH content exhibits a significant dose-dependent decline along with the increased ADM concentration in group with pretreatment of DEM, while the GSH content didn't show significant changes in group without DEM pretreatment along with the increased ADM concentration.

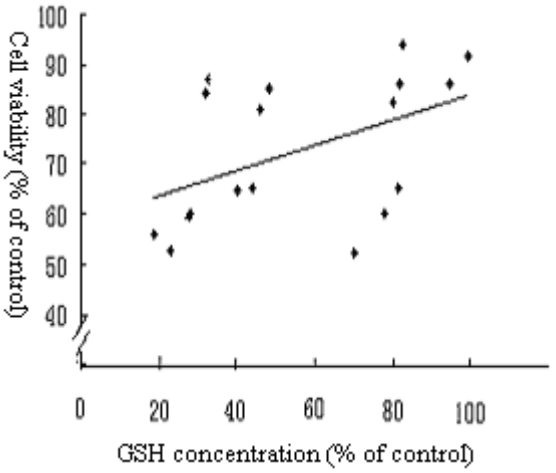

Fig 8 Correlation between the Cell Viability of MCF-7/ADM and Its Intracellular GSH Concentration （r=0.90, P＜0.05）

Discussion

Apparent Oxidative Deviation of GSH/GSSG Status in Plasma of Cancer Patients, but Potential Reductive Deviation in Tumor Tissues

Our data on GSH/GSSG status in plasma of cancer patients showed a obvious pro-oxidant deviation compared with healthy subjects, which was consistent with previous reports[7,8,9,10]. The oxidative stress may come from increased reactive oxygen species (ROS) generation by tumor cells. In 70 years ago, a landmark research by Warburg[11] pioneered the fact that the mitochondrial defects of malignant cells played an important role in the development and progression of cancer, which rendered the cancer to satisfy their energy needs by producing a large portion of their ATP through glycolysis rather than through oxidative phosphorylation, but the mitochondrial defects would compromise the normal electron flow and result in an increase generation of superoxide radicals, which are subsequently converted into other ROS. Studies in Zhou Y et al have demonstrated that primary leukemia cells from patients with chronic lymphocytic leukemia contain significantly higher levels of cellular ROS compared to normal lymphocytes [12].

The increased ROS generation may also come from anticancer immune response and hypoxic microenvironment in solid tumors, mounting experimental and clinical evidences has demonstrated a obvious increased production of ROS in numerous pathophysiological settings including immune reaction、inflammation、hypoxia etc[13,14,15].

The pro-oxidant deviation of GSH/GSSG status in plasma reflected a general oxidative stress in cancer patients. The GSH/GSSG status in cancer tissue assayed by this study, however, displayed a significant higher level of GSH and GSH/GSSG ratio in tumor tissues than their counterparts of paratumor tissues, although the tumor cells produced numerous ROS, the significant increased level of GSH maintains an apparent reductive deviation in tumor tissues, this bifurcation of GSH/GSSG status in cancer tissue and plasma may represent a somewhat unique characteristics of malignant cells.

Oxidative stress possesses ambilateral functions, mild-moderate oxidative stress is able to promote proliferation of tumor cell. Lots of signal pathway such as endothelial growth factor receptor, platelet derived growth factor receptor and MAPKs, which are related with proliferation, differentiation and migration of cells, are all redox-sensitive. The increase in endogenous ROS thus provides a constant stimulus for cell proliferation, and most importantly, may cause further damage to both mtDNA and nDNA, leading to cancer development, genetic instability, and disease progression[1,2].

On the other hand, oxygen free radicals are potent inducer of apoptosis for tumor cells [12,16]. One of the anticancer mechanisms of radiotherapy and chemotherapy was the production of ROS to kill tumor cells. The significant increased GSH provides the tumor cells a strong anti-oxidative stress and anti-apoptosis capacity, this nature may be even related with its drug resistance mechanisms.

So, the bifurcation of GSH/GSSG status in plasma and cancer tissues may render the malignant cells a great advantage over the double-edged effects of ROS, and a potent growth/survival potentials.

Proliferation-promoting Effect of Malignant Cells on Endothelial Cells is Partial GSH/GSSG Status-dependent

Since Dr. Judah Folkman first proposed the hypothesis in 1971 that tumor growth was angiogenesis dependent[18], the role of angiogenesis in tumor growth and progression has been firmly established, the tumor's angiogenesis is widely recognized been deeply involved in tumor progression and metastasis[19]. The process of tumor angiogenesis is thought to be primarily determined by the pro-angiogenic regulators released from malignant cells, such as hypoxia inducible factor-α, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) etc, and considerable effort has been directed to anti-angiogenic therapy against these angiogenic growth factors and their receptors to treat human cancers[20].

But how does the endothelial cell function in its own proliferation? In the present study, the proliferation of endothelial cells was inspired by tumor conditioned medium KCM in a dose-dependent manner, which suggested the existence of pro-angiogenic substances

secreted by tumor cells. In this regard, various angiogenic growth factors such as HIFα、PDGF, VEGF, bFGF, etc have been frequently found in high concentrations in tumors, it thus is not surprising why the KCM showed significant promoting effect on proliferation of endothelial cells. Meanwhile, the present data also showed that the KCM treatment enhanced GSH content in endothelial cells, but did not change the GSH/GSSG ratio, which suggested maintenance of a constant redox status of GSH/GSSG by endothelial cells under the KCM stimulation.

Treatment of endothelial cells with BSO, a specific inhibitor of de novo GSH synthesis, reduced the endothelial intracellular GSH content down to approximately 6.5% of control, which but just showed a mild impact on cell viability. Under the background of depletion of GSH by BSO, the proliferation-promoting effect on endothelial cells by KCM was totally reversed even in a negative dose-dependent manner, the endothelial viability showed a downhill trend along with the increasing concentration of KCM, and the redox state of GSH/GSSG couple deviated to more and more oxidized with the increase of concentration of KCM. Correlation analyzing of proliferating activity of endothelial cells with its intracellular GSH/GSSG redox status displayed a close relationship($r=0.865$，$P<0.01$).

These results showed that if the endothelial redox state of GSH/GSSG was maintained constant, the malignant cells stimulated endothelial proliferation, and GSH synthesis; But if inhibiting the GSH synthesis of endothelial cells, the stimulating effects of the malignant cells on endothelial cells was totally reversed, the effects of malignant cell on endothelial cell became anti-survival on endothelial viability and pro-oxidant on GSH/GSSG redox status. In summary, our data showed that the proliferation-promoting effect of malignant cells on endothelial cells exhibits a trait of partial dependent on GSH/GSSG redox status

Increased GSH Level in Malignant Cell Correlated with It's Drug Resistance

Several lines of evidence have indicated that block of GSH synthesis could reverse the drug resistance in assorted cancer cells[17,21], the effect may involve the multidrug-resistance- associated protein (MRP), which is a plasma membrane glycoprotein that can lower intracellular drug concentration by transport a complex of drugs and GSH out of cells. Attempts have been made to modify drug sensitivity with BSO, the inhibitor of GSH synthesis, and this compound has even been tested in some humans[22]

But the dependence of drug resistance on intracellular GSH was not conclusively ascertained. Franzini M et al[23] recently reported that the cancer cells transfected by GGT, the gamma-glutamyltransferase , which is regarded as critical for the maintenance of intracellular levels of glutathione, exhibited reduced sensitivity to cisplatin, but a decrease rather than an increase of intracellular GSH levels.

We show here that the intracellular GSH content in breast cancer cells with adriamycin resistant (MCF-7/ADM) is much higher than adriamycin sensitive cells(MCF-7/S); depletion of intracellular GSH by DEM increases the adriamycin sensitivity in both MCF-7/ADM and MCF-7/S cells; and the adriamycin exerted a significant dose-dependent reducing effect on intracellular GSH content in DEM pretreated cells, the decrease of intracellular GSH level was even stronger than the simple added effects of DEM with adriamycin. All the data suggested that the intracellular GSH level was an important factor in regulating the drug-sensitivity, which supported the view of the dependence of drug resistance on intracellular GSH level. This result was also in accordance with our finding in the first part, that a significant higher level of GSH and GSH/GSSG ratio was found in tumor tissues, the potential reductive deviation in tumor tissues renders the tumor cells a privilege over oxidative stress and cytotoxic drugs.

In summary, the present study indicated that the GSH/GSSG redox status is an important determinant in regulating some of the tumors biological properties, further investigation for regulating ways of GSH/GSSG redox status may be very worthful in anticancer study.

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Factors influencing agricultural extension officers' knowledge on practice and marketing of organic agriculture in North West Province, South Africa

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Abstract: This paper examines the factors influencing agricultural extension officers' knowledge on practice and marketing of organic agriculture in North West Province, South Africa. A simple random sampling technique was used to select 20 percent extension officers from which data were collected with a structured questionnaire and analyzed using frequency counts, percentages and multiple regression analysis. The results revealed that extension officers had a wide range of knowledge levels regarding marketing of organic agriculture as all the items had at least 55 percent. Significant determinants of knowledge on practice and marketing of organic agriculture were gender (t= 2.46), age (t= - 1.73), educational level (t = 1.75), working experience (t = - 1.71), job location (t = 2.72) and sources of information (- 3.02) . The results have several implications for training and educating extension officers in organic agriculture issues. [Life Science Journal 2010;7(3):91-98]. (ISSN: 1097-8135).

Keywords: Organic agriculture, extension officers, knowledge, marketing, South Africa

1. Introduction

Agriculture has reached the limits of available natural resources such that future increases in agricultural production and rural income must derive from intensification, rather than area expansion and exploitation of additional natural resources. Agriculture itself is changing and changing the quantity and nature of information farmers need. Information needs increase both with greater commercialization of agriculture and in light of the innovations that are likely to prove important in the future. Knowledge, information, skills, technologies, and attitudes are to be explored for the sustainable intensification of agriculture and rural development(FAO and World Bank, 2000). Agricultural extension plays an important catalytic role in agricultural and rural development as it brings the farming community information and new technologies that can be adopted to improve production, incomes and standards of living. Agricultural extension provides a channel by which farmers' problems are identified for research and the modification of agricultural policies. The extension system also organizes farmers into functional groups in order to gain access to production resources such as credit, inputs, marketing services and information. Agricultural extension programmes are very diverse from an international perspective as most are managed as public sector agencies and some nongovernmental organizations (NGOs while many private firms and private organizations conduct extension programs. An equally important variation occurs in the skill and competence of extension staff (Oladele and Sakagami, 2004). In competitive production environment, occasioned by the globalization policy, extension services must be oriented to markets and overcome the exclusive focus on production that ignored market demand and profitability as was the constraint of many past extension programs. Varied extension services are needed to help farmers remain competitive and profitable, diversify production, produce for niche markets, and move to higher-value products and more value-added production.

Agricultural extension is the most important source of information to farmers in most African countries (Agbamu, 2002) and play significant role in affecting farmers' adoption of innovations (Van den Ban and Hawkins, 1998). The effectiveness of extension service delivery is critically dependant on the knowledge of extension officers on the various agricultural innovations they disseminate to farmers, thus, the purpose of this paper is to identify factors that determine extension officers' knowledge levels on practice and marketing of organic agriculture. This is based on the fact that the knowledge of extension officers will influence their attitude and the kind of awareness they create on organic agriculture among farmers. Long and Sworzel (2007) noted that the mission of extension services is to provide research based information, educational programs and technology on farmers' needs and enabling them to make informed decisions about their economic, social and cultural well-being.

The goal of organic farming is to give priority to long-term ecological health, such as biodiversity and soil quality, rather than short-term productivity gains (IFOAM, 2006). Organic farming is a widely respected approach to overcoming the negative impacts of the Green Revolution on soil, air, water, produce, landscape, and humans worldwide. Organic farming was and is constantly being developed by farmers, scientists and concerned people all over the world. A central element of the organic farming method is the efficient use of on-farm and local resources like farmyard manure, indirect crop protection and local seed. Organic farming promotes the powers of self-regulation and resistance which plants and animals possess naturally and therefore, organic agriculture is not just a solution for more affluent countries but applied in every climatic region. In poorer countries especially, it can contribute to purposeful socio-economic and ecologically sustainable development (Kilcher, 2002; Mc Neely and Scherr, 2002; and Yussefi and Willer, 2003). Organic farming, therefore, has become an issue of public concern, but it has also become a big business. This business is being met by legislation and governmental standards on organic farming which include rules for processing, trading, monitoring, and certifying agricultural produce.

South Africa is not only self-sufficient in virtually all major agricultural products, but in a normal year it is also a net food exporter. However, with very low average rainfall and high variability within and between seasons, its agriculture is vulnerable to the effects of drought (FAO, 2006). However South Africa has been described as a fertile territory for GM crops and well ahead of the rest of Africa when it comes to biotech. Already more than 200 permits for field trials have been issued and three GMO crops are commercially available. This is predicated on the fact that agriculture is dominated by a small number of large-scale farms that are highly integrated into the commercial seed market dominated by Monsanto, Pioneer Hi-Bred and Sakata. The country's public research institutions, which have carried out biotech research since the apartheid years, are in the midst of a privatization blitz and the biosafety and intellectual property rights legislation in place favors the biotech industry.

Despite the above description of South African agriculture with reference to the prevalence of biotech and GMO based cropping, South Africa has had an organic sector for many years, although it has grown in 'fits and starts'. It comprises 250 certified farms and 45,000 hectares of certified organic land, which account for 0.05 % of the country's total agricultural area (IFOAM and FiBL, 2006). South Africa is one of the two countries in Africa with a robust domestic market, although it is underdeveloped and there are few, if any, price premiums for organic products. Most organic growers therefore look to Europe as their outlet area (IFOAM, 2003). South Africa is also a market destination for organic produce from nearby southern African states, particularly Mozambique. Organic agriculture is generally assumed to cater to a luxury niche whose customers can afford to shop in health food, rather than hard discount, stores however the reality today is that organic supply is now the world's fastest-growing food sector, increasing at 15 percent a year over the last decade and worth some 40 billion dollars in 2006. Consumer studies too reveal that organic buyers are not so much better-off in status as generally more aware of food issues.

Available information and the sources of such information have been one of the critical factors affecting adoption rates of innovations among farmers (IFOAM, 2003). Van den Ban and Hawkins (1998), Rogers, (2003) and Fuglie and Kascak (2001) illustrated quantitatively the positive role that extension officers play in diffusing agricultural innovations. Oladele (2005) reported that farmers indicated that the most dependable source of information is agricultural extension agent. OFRF (1999) and Wheeler (2007), noted that organic farmers have often complained about agricultural professionals' negative attitude about, and lack of knowledge of, organic farming. Anecdotal evidences also suggests that organic farmers often state that if professionals remained open-minded and took the time to learn more about organic farming then they would be more likely to think favourably about it as a farming system. Wheeler (2008) asserted that conservative or negative attitudes within the agricultural scientific and academic community are seen as significant barriers to research, extension support and hence the adoption of organic agriculture by farmers. There have been many studies on the consumer side with respect to organic agriculture, but there have been no studies exploring the determinants of knowledge level of extension officers on organic agriculture. This paper attempts to isolate these determinants as this would in reveal the knowledge level of the extension officer and how they can educate their farmers on issues related to organic agriculture.

2. Materials and Methods

The study was carried out in North West province, South Africa. The study population was all extension officers (200) in the province. Extension officers in this study are employees of the Department of Agriculture that have the responsibilities of providing information to farmers on all aspect of farming as well as advisory

services. A simple random sampling technique was used to select 20 percent extension officers from which data were collected. A structured questionnaire was developed based on the study objectives and related literature which was divided into three parts. The first sought demographic characteristics of extension officers and the second elicited information on knowledge on climate change which was anchored as True (2) or False (1) on a scale with 93 items covering sections on practice, health, environment and marketing. The questionnaire was faced validated by lecturers from the Department of Agricultural and Extension at North West University, and their suggestions were incorporated into the instrument before data collection. The questionnaire had a reliability coefficient of 0.87 determined by the split-half technique. Data obtained was analyzed with the Statistical Package for Social Sciences (S.P.S.S) using frequency counts, percentages, means and linear multiple regression analysis.

A linear regression model was used to isolate the determinants of knowledge level of extension officers on organic agriculture. A probit model is appropriate when the dependent variable to be evaluated is dichotomous. The relationship between the probability of a variable Pi and its determinants q is given as:

$Pi = \beta qi + \mu i$ …… (1)

Where Pi=1 for Xi>Z; i=1,2 ....., n; qi is a vector of explanatory variables and β is the vector of parameters. The probit model computes the maximum likelihood estimator of β given the non-linear probability distribution of the random error μi.

When the dependent variable takes more than two values and these two values have a natural ordering, the use of an ordered probit is indicated and estimated using the maximum likelihood method.

Most studies on organic farming have used probit or logit models while some have used ordered probit specification. In the probit model the discrete dependent variable Y is a rough categorization of a continuous, but unobserved variable Y^*. If Y^* could be directly observed then standard regression methods would be used (such as assuming that Y^* is a linear function of some independent variables, for example:

$Y^* = \beta_1 X_{1i} + \ldots\ldots\ldots \beta_j X_{ji} + ui$ ……(2)

In this study, Y^* is the knowledge level of extension officer on organic which is observable with a lower and upper limit, then the use of linear regression is justified. The model is therefore specified as

$Y = f(X_1, X_2, \ldots, X_{10})$ …….(3)

The actual model specification is:
Knowledge of organic farming
$= \beta_0 + \beta_1$Gender $+ \beta_2$Age $+ \beta_3$Religion $+ \beta_4$Educational level $+ \beta_5$Studying for higher degree $+ \beta_6$Working experience $+ \beta_7$Rural-urban background $+ \beta_8$Job location $+ \beta_9$Information sources $+ \beta_{10}$No of farmers covered $+ \beta_{11}$Living in job area $+ u$.

3. Results

Table 1 presents the personal characteristics of extension officers in North West Province, South Africa.

Table 1. Personal characteristics of extension officers

Personal Characteristics	Description
Gender	Predominantly male 52.5 %
Age	Mean = 42.5 years
Marital Status	72.5 %Married
Religion	Predominantly Christianity 82.5%
Educational level	Predominantly Diploma 85%
Household size	Mean = 4.2 persons
Working experience	Mean = 14 years
Living in job location	Predominantly Yes 85%
Job designation	Predominantly extension officer 43%

In Table 2, the results of extension officers' knowledge on marketing of organic agriculture were presented.

The results of extension officers' knowledge on practice of organic agriculture were presented in Table 3.

The result of multiple regression analysis on the determinants of extension officers' level of knowledge on the practice and marketing of organic agriculture is shown in Table 4.

Table 2: Knowledge on marketing of organic agriculture

Statements	True	False
Consumers, who want to buy organic products will support organic farming	37(92.5)	3 (7.5)
Experts in standard setting, monitoring, control, and certification will support organic farming	32(80)	8(20)
Consumption and environmental responsibility will enhance support for organic farming	29(72.5)	11(27.5)
Organic agriculture will lead to improvements to external factors.	22(55)	18(45)
The closer the farmers are to the market and information centres, the better the tendency to adopt organic farming.	28(70)	12(30)
Market for organic products is poorly developed and organic products are either home delivered and or sold in the few supermarkets and hotels.	33(82.5)	7(17.5)
Organic agriculture will ensure the quality of the products	26(65)	14(35)
Consumers who sometimes buy organic food are more concerned with health reasons.	29(72.5)	11(27.5)
Organic agriculture will ensure the economic value of food	28(70)	12(30)
Product quality characteristics affect consumers' preferences for organic food;	24(60)	16(40)
Religion influences Organic agriculture practices and marketing	26(65)	14(35)
Organic agriculture will address those that believe that a price premium on a product signals a better product	23(57.5)	17(42.5)
Organic foods are cheaper	23(57.5)	17(42.5)
Marketability of organic agriculture product is low in RSA	25(62.5)	15(37.5)
Organic methods often require more labour, providing rural jobs but increasing costs to urban consumers.	29(72.5)	11(27.5)
Organic farming yields more and uses less land for the same output level.	31(77.5)	9(22.5)
Organic production management system offered a real and affordable means to break out of poverty and obtain food security in marginal areas."	27(67.5)	13(32.5)
Organic agriculture saves farmers significantly from expensive insecticides, fungicides and other pesticides.	26(65)	14(35)
Consumers who recognise the greater food value of organic produce will be willing to pay premium prices for it.	31(77.5)	9(22.5)
Food quality is more important than price.	37(92.5)	3(7.5)
Organic food buyers were more health conscious, and did not trust conventional food.	34(85)	6(15)
The motivation for buying organic was the absence of contaminants or health reasons.	35(87.5)	5(12.5)
Availability and price will influence actual purchase of organic foods	35(87.5)	5(12.5)

Table 3. Knowledge on practice of organic agriculture

Statements	True	False
Organic farming is a form of agriculture that relies on natural inputs for crop and animal production	34(85)	6 (15)
Organic farming requires considerably more skill to farm -	34(85)	6 (15)
Organic agriculture promotes the health of the agro-ecosystem related to biodiversity, nutrient biological cycles, soil microbial and biochemical activity.	34(85)	6 (15)
Organic agriculture sustains the health of soils, ecosystems and people.	40 (100)	

Organic agriculture relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects.	34(85)	6 (15)
Organic Agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them.	40(100)	
Organic Agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible.	39(97.5)	1(2.5)
Organic Agriculture should build on relationships that ensure fairness with regard to the common environment and life opportunities	32(80)	8(20)
Organic Agriculture should be managed in a precautionary and responsible manner to protect the health and well-being of current and future generations and the environment.	39(97.5)	1(2.5)
Farmers, who are willing and able to adopt organic land use systems; will support organic farming	40(100)	
Organic agriculture will lead to improvements in availability of food	35(87.5)	5(12.5)
Organic agriculture will lead to improvements to natural capital	26(65)	14(35)
Organic agriculture will lead to improvements to social capital	37(92.5)	3(7.5)
Organic agriculture will lead to improvements to human capital	24(60)	16(40)
Organic agriculture will lead to improvements to physical capital.	27(67.5)	13(32.5)
Organic agriculture will ensure the nutritional value of farm products	31(77.5)	9(22.5)
Food insecurity will not enhance support for organic agriculture	23(57.5)	17(42.5)
Organic agriculture is based on the principle of fairness	25(62.5)	15(37.5)
Organic agriculture is not a popular practice in South Africa	33(82.5)	7(17.5)
Organic agriculture is in practice in South Africa	23(57.5)	17(42.5)
Organic agriculture involves the use of indigenous methods	22(55)	18(45)
Organic farming requires more land holds only for cash crops.	37(92.5)	3(7.5)
Organic farming enhances crop-livestock integration	34(85)	6 (15)
Effective organic pest control requires a thorough understanding of pest life cycles and interactions.	26(65)	14(35)
Weeds are controlled mechanically, and through the use of cover crops and mulches in organic farming	26(65)	14(35)
Organic agricultural methods are internationally regulated and legally enforced by many nations,	25(62.5)	15(37.5)
Organic farmers also use certain processed fertilisers such as seed meal, and various mineral powders such as rock phosphate and greensand, a naturally occurring form of potash.	37(92.5)	3(7.5)
Organic pest control involves allowing for an acceptable level of pest damage, intro-ducing beneficial organisms,	31(77.5)	9(22.5)
Organically grown compete better with weeds that are present.	33(82.5)	7(17.5)
Organic farming is more labour intensive than chemical/mechanical agriculture	35(87.5)	5(12.5)
Organic farming requires greater interaction between a farmer and crops for observation and timely intervention	32(80)	8(20)
Organic growers do not use genetically modified or engineered food crops,	27(67.5)	13(32.5)
Organic agriculture yields decline over time	27(67.5)	13(32.5)
Organically grown plants are more droughts tolerant.	32(80)	8(20)
Organic agriculture will address those that are concerned with chemical hazards among farm workers	31(77.5)	9(22.5)
A healthy plant grown organically in properly balanced soil resists most diseases and insect pests.	26(65)	14(35)

Table 4. Multiple regression analysis of determinants of knowledge on practice and marketing

	Practice		Marketing	
	Reg Coeff/ SE	t	Reg Coeff/ SE	t
(Constant)	152.926 (31.310)	4.884	47.426 (12.961)	3.659
Gender	32.2(13.06)	2.46	10.3(5.36)	1.92
Age	-.986(.569)	-1.732	-.310(.236)	-1.316
Religion	-2.718(2.629)	-1.034	-1.566(1.088)	-1.439
Educational level	0.75(0.43)	1.75	0.44(0.22)	1.98
Studying for higher degrees	-5.064(8.335)	-.608	-4.337(3.450)	-1.257
Working experience	-.550(.321)	-1.714	-.331(.133)	-2.490
Rural-urban background	9.513(10.772)	.883	5.796(4.459)	1.300
Job location	19.499(7.179)	2.716	7.809(2.972)	2.628
Number of farmers covered	-.099(.617)	-.161	.123(.256)	.482
Sources of information	-11.33 (3.74)	-3.02	-2.79(1.26)	-2.20
F	2.47		3.22	
p	0.03		0.03	
R	0.59		0.69	
R square	0.35		0.48	

4. Discussions

The table shows that extension officers were predominantly male (52.5%) with the mean age of 42.5 years, married (72.5%) and 82.5% were Christians. In terms of the educational level, 85% of the extension officer had Diploma as their qualification and a mean of 14 years as working experience. Table 1 further revealed that there was a mean of 4.2 persons per household and 85% live in their job location, rural or peri-urban notwithstanding. In terms of job designation 43% were extension officer with the remaining percentage in higher positions and cadre. This agrees with the findings of (Zwane, 2009) who reported that extension officers in Limpopo province of South Africa were mainly males, between 40 t0 49 years, and had Diploma as their educational qualification. Bembridge(1991), also reported similar findings in terms of the personal characteristics of extension officers in South Africa.

In Table 2, the results revealed that extension officers had a wide range of knowledge levels regarding marketing of organic agriculture as all the items had at least 55 percent. Prominent items with right responses were consumers, who want to buy organic products will support organic farming (92.5%); food quality is associated with price(92.5%) the motivation for buying organic was the absence of contaminants or health reasons (87.5%) and availability and price will influence actual purchase of organic foods(87.5%).However extension officers had low knowledge on item s such as organic agriculture will lead to improvements to external factors (45%); product quality characteristics affect consumers' preferences for organic food(40%); organic agriculture will address those that believe that a price premium on a product signals a better product(42.5%) and organic foods are cheaper(42.5%). The results have several implications for training and educating extension officers marketing of organic agriculture as[4] reported that extension officers often work

reactively rather than proactively because of the large number of clients and the small number of extension agents.

From Table 3, the results revealed that extension officers had a wide range of knowledge levels regarding practice of organic agriculture. No respondents answered all of the knowledge test questions incorrectly, nor did any respondents answer all questions correctly. Items with high proportion of right responses were organic agriculture sustains the health of soils, ecosystems and people(100%); organic agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them (100%); farmers, who are willing and able to adopt organic land use systems will support organic farming (100%); farmers, who are willing and able to adopt organic land use systems; will support organic farming(100%); organic agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible (97.5%) and organic agriculture should be managed in a precautionary and responsible manner to protect the health and well-being of current and future generations and the environment (97.5%) . it can be inferred from the results that extension officer shad more knowledge on practice than marketing of organic agriculture. Conversely, extension officers' recorded low knowledge on item s such as organic agriculture involves the use of indigenous methods (45%); organic agriculture will lead to improvements to human capital (40%); food insecurity will not enhance support for organic agriculture (42.5%) and organic agriculture is in practice in South Africa (42.5%).

In table 4, the independent variables were significantly related to knowledge levels of extension officers on practice and marketing of organic agriculture with F values of 2.47 and 3.22, $p < 0.05$ respectively. Also, R values of 0.59 and 0.69 showed that there was a strong correlation between independent variables and knowledge levels of extension officers on practice and marketing of organic agriculture respectively. The result further predicted 35 and 48 percent of the variation in knowledge levels of extension officers on practice and marketing of organic agriculture. Significant determinants of knowledge on practice of organic agriculture were gender (t= 2.46), age (t= - 1.73), educational level (t = 1.75), working experience (t = - 1.71), job location (t = 2.72) and sources of information (- 3.02) . Similarly, significant determinants of knowledge on marketing of organic agriculture were gender (t= 1.92), educational level (t = 1.98), working experience (t = - 2.49), job location (t = 2.62) and sources of information (- 2.20). It implies that the more there are more male extension officers, with high educational level and residing in their job location the higher the knowledge level on practice and marketing of organic agriculture. However, as extension officers get older, with few years of working experience and poor exposure to sources of information the lower the knowledge on practice and marketing of organic agriculture. The trend and the sign of the coefficients could be due to the fact that there is male dominance of the extension service delivery in the study area; with majority of the extension officer having diploma as their education al qualification and residing in their job location. The acquisition of a higher level of education would improve the competence and skills of the extension officers and there would be more interaction and awareness of farmers' enterprises when they reside within their job location. Extension officers with few years of working experience would not have mastered the diversity of interests in the farming enterprises among farmers; while poor exposure to sources of information such as workshops and training would impact negatively on the knowledge level of extension officers. Wheeler[17] found that similar variables influence agricultural professional views on organic agriculture in Australia.

However South Africa has been described as a fertile territory for GM crops and well ahead of the rest of Africa when it comes to biotech. Despite the description of South African agriculture with reference to the prevalence of biotech and GMO based cropping, South Africa has had an organic sector for many years and is increasingly stronger. Agricultural extension is the most important source of information to farmers in most African countries and play significant role in affecting farmers' adoption of innovations. The effectiveness of extension service delivery is critically dependant on the knowledge of extension officers on the various agricultural innovations they disseminate to farmers. The study showed that extension officers in North west Province, South Africa were predominantly male, with the mean age of 42.5 years, married, Christians, had Diploma as educational qualification and a mean of 14 years as working experience. Extension Officers had a wide range of knowledge levels regarding the practice and marketing of organic agriculture as all the items had at least 55 percent. Significant determinants of knowledge on practice and marketing of organic agriculture were gender, age, and educational level, working experience, job location and sources of information. The results have several implications for training and educating extension officers in organic agriculture issues. The results of this study add to the existing literature on the determinants of knowledge levels among extension officers and thus will inform educational and training policy on agricultural innovations in

order to improve the competence of extension officers.

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Socio-economic Determinants of Job Satisfaction among Extension Officers in North West Province South Africa

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Abstract: This paper examines the socio-economic determinants of job satisfaction among extension officers in North West Province South Africa. This is predicated on the fact that the current implementation of the Extension Recovery Plan (ERP) in all the nine provinces in the South Africa to bring about agricultural improvement through effective extension services delivery is among other things dependent on job satisfaction among extension officers. A simple random sampling technique was used to select 40 extension officers and data were collected with a structured questionnaire and analyzed using frequency counts, percentages and multiple regression analysis. The results show that most of extension officers (82.5%) were males, between 40 to 45 years, married (85%0 and 87.5% had Diploma as educational qualification. Also, 54% had between 4 to 6 persons as household size, while 75% were Christians. Majority of the extension officers live in their job area (82.5), while 80% covered at least 3 communities and 3 farmers group. In terms of number of farmers covered, only 40%of the extension officer covered more than 500 farmers and 45% travelled more than 40 km to reach their farmers. Prominent areas of satisfaction among extension officers were research policies (3.77) work exposure (3.37) identifying farmers' problems (3.02) and opportunities to advance education (3.00). Significant determinants of job satisfaction were gender (t = 2.31), marital status (t = -2.27), working experience (t = 2.60), living in job area (t = -3.05), number of farmers covered (t = 2.00) and distance to farmers (t = -2.11). The study therefore recommends that policy makers and extension managers should pay attention to the items indicated for satisfaction and dissatisfaction by the extension officers with a view of boosting their morale for a higher level of performance. [Life Science Journal 2010;7(3):99-104]. (ISSN: 1097-8135).

Key words: Job satisfaction; job performance; motivation; extension officers; South Africa

1. Introduction

In many developing countries agricultural development is hinged on extension services by helping farmers to identify and link with research on their production problems. They also provide awareness on opportunities for improvement of farm yields leading to increased income and better standard of living (Van den Ban and Hawkins, 1998, Agabmu, 2002). Long and Sworzel (2007) noted that the mission of extension services is to provide research based information, educational programs and technology on farmers' needs and enabling them to make informed decisions about their economic, social and cultural well-being. Santucci (2002) noted that most farmers in African countries depend on public extension workers for information. Sadique (1981) described the extension agent as a professionally trained social worker, an organizer, a planner, a facilitator and a consultant. Adams (1982) referred extension agents as advisers, middlemen and managers at the cell or circle level. They are thus (a) analyst – interpreting situation for his clients (b) Advocator-choosing the best method among alternatives (c) Innovator-creating new ideals to satisfy a particular need of client (d) Advisor-making available alternative to a given situation. The role of the village extension agent is frequently stressed since of all extension staff functions to farmers, critical teaching and persuading fanners to adopt production recommendations and feeding back to the extension and research services, information on actual-farm production conditions and constraints and farmers reactions to recommended practices (Benor and Baxter, 1984).

The effectiveness of extension is dependent upon the motivation of its employees (Chesney, 1992). Knowing what motivates employees and incorporating this knowledge into the reward system will help extension mangers identify, recruit, employ, train, and retain a productive workforce. At one time, employees were considered just another input in production process until the Hawthorne studies, which began the human relations approach to management, whereby the needs and motivation of employees become the primary focus of managers (Bedeian, 1993).

The extension workforce has attracted individuals of diverse and different characteristics such as age, gender, working experience, educational qualification and marital status among other characteristics which have been found to be associated with job satisfaction either positively or negatively. Schermaerhon et al (1995) defined job satisfaction as the degree to which an individual feels

positively about various facets of the job task, the work setting and relationship with co-workers. Employer should build into employees' characteristics that create satisfying conditions. However because people respond differently to the same job, employers must take into consideration both job characteristics and the work context of the job itself. Scott et al (2005) reported that significant relationship exist between job satisfaction and gender. Nestor and Leary (2000) found no relationship between gender and job satisfaction. Riggs and Beus (1993) found that as the number of areas of responsibility increased for female agents, job satisfaction increased as well. Also female agents who had fewer areas of responsibility and fewer areas of responsibility and fewer children living at home were more satisfied. Employees work harder and perform better if satisfied with their jobs, knowing the factors related to agents' job satisfaction can help prevent staff frustration and low job satisfaction (Watanabe, 1991). The extent to which people are satisfied with their work has been of enduring research interest. An increasing concern with the meaning of work and the belief that the degree of satisfaction at work is related to aspects of work behavior such as productivity, absenteeism, turnover rates and intention to quit, have prompted the growth of a vast research literature on job satisfaction. The most important information to have regarding employees in organizations is a validated measure of their level of job satisfaction. Although job satisfaction has been viewed in a number of different ways, most definitions agree that it is a multidimensional concept (Koustelios, 2001). Dawis and Lofquist (1984) defined job satisfaction as the result of the worker's appraisal of the degree to which the work environment fulfills the individual's needs. These definitions are similar to others viewing satisfaction as the degree of an employee's affective orientation toward the work role occupied in the organization.

Department of Agriculture, (2009) reported that currently, the Extension Recovery Plan (ERP) is being implemented in all the nine provinces in the South Africa. This is predicated on the fact that agricultural improvement in South Africa, especially among small scale and resource-poor farmers, requires a major effort to improve the quality of extension services available to farmers. The implementation of ERP was based on 5 pillars namely ensuring the accountability and visibility of extension, promotion of professionalism and improvement of image, re-skilling and reorientation of extension, provision of ICT infrastructure and other resources and recruitment of extension personnel. The foregoing has several implications on extension delivery s and consequently requires that extension officers are sufficiently satisfied and motivated for effective job performance. The purpose of this study was to identify socio-economic determinants of job satisfaction among extension officers in North West Province South Africa.

2. Materials and Methods

The study was carried out in North West province, South Africa. The study population included all extension officers (200) in the province. A simple random sampling technique was used to select 40 extension officers from which data were collected. A structured questionnaire consisting of 34 job satisfaction and 10 personal characteristic items was administered to extension officers. Job satisfaction was measured on a 5-point Likert type scale of not satisfied (1), least satisfied (2), fairly satisfied (3), almost satisfied (4), and most satisfied (5). The questionnaire was face validated by lecturer in the Department of Agricultural Economics and Extension of the North West University and extension professionals from the Department of Agriculture, South Africa. The reliability coefficient for the questionnaire was 0.85. Data collected were analyzed using Statistical Package for Social Sciences (SPSS), with frequency counts, percentages, means, standard deviation and multiple regression analysis.

3. Results

Table 1 shows demographic characteristics of extension officers in North West Province South Africa.

Table 1. Demographic characteristics of extension officer

Variables	Frequency	Percentages
Gender		
Male	33	82.5
Female	7	17.5
Age		
Less than 40	5	12.5
40-45	19	47.5
45-50	16	40.0
Marital status		
Married	34	85.0
Divorced	3	7.5
Single	3	7.5
Household size* n= 37		
1-3	10	27
4-6	20	54.05
Above 6	7	18.95

Religion		
Christianity	30	75.0
Free thinkers	10	25.0
Educational level		
Diploma	35	87.5
B.Sc	5	12.5
Studying for higher degree		
Yes	35	87.5
No	5	12.5
Working Experience		
Less than 10 years	**3**	7.5
10-20 years	27	67.5
Above 20 years	10	25.0
Living in job area		
Yes	33	82.5
No	7	17.5
Job Designation		
Agricultural Technician	18	45.0
Senior Agricultural Technicians	3	7.5
Extension officers	4	10.0
Chief Agricultural Technicians	10	25.0
Senior Extension officers	5	12.5
Number of communities covered		
1-3	8	20.0
4-6	19	47.5
Above 6	13	32.5
Number of farmers groups covered		
1-3	8	20.0
4-6	15	37.5
Above 6	17	42.5
Number of farmers covered		
Less than 200	5	12.5
200 - 500	19	47.5
Above 500	16	40.0
Distance to farmers		
Less than 40 km	5	12.5
40-100 km	17	42.5
Above 100 km	18	45.0

Table 2 shows the mean and standard deviation of 34 items on job satisfaction by extension officers which were rated on a 5-point Likert type scale of not satisfied (1), least satisfied (2), fairly satisfied (3), almost satisfied (4) and most satisfied (5). The actual mean was 3 due to the rating scale and a mean of greater than 3 denoted satisfaction, while a mean less than 3 denoted dissatisfaction.

Table 2. Job satisfaction index among extension officers

Items	Mean	SD
Qualification for job	2.90	1.19
In-service training	2.77	1.29
Work exposure	3.37	1.05
Lack of motivation	2.32	1.18
Direction by supervisors	2.33	1.14
Research policies	3.77	0.89
Political problems	2.10	0.95
Identifying farmers' problems	3.02	1.29
Feeding back farmers' problems to research	2.52	0.96
Communicating recommended practices	2.65	1.27
Liaison with other agencies	2.60	0.84
Job specialization	2.07	1.04

Financial support for self and family	2.15	1.00
Job security	2.60	0.95
Job autonomy	2.40	1.27
Operating supplies and materials	2.22	1.34
Transportation	2.70	1.22
Availability of experimental land	1.90	1.05
Work equipment and tools	2.10	0.87
Availability of labour/technical help	2.45	0.95
Quality of labour/technical help	2.37	1.00
Library facilities	2.15	1.12
Opportunities to advance education	3.00	1.26
Opportunities to gain professional recognition	2.95	1.15
Opportunities to publish findings	2.00	1.01
Management reputation for professional achievement	2.30	0.99
Management control of operations	2.32	0.85
Flexibility and initiative	2.67	1.07
Rewarding system	1.97	1.09
Relationship among professionals and administrative staff	2.45	0.90
Continuity of programme	2.07	1.02
Clear statement of project embarked upon	2.10	1.25
Budgeting	1.90	1.00
Sanctions	1.90	1.12

The result of multiple regression analysis of relationships between extension officers' socio-economic characteristics and job satisfaction were presented in Table 4.

Table 3: Multiple regression analysis of determinants of job satisfaction among extension officers

	B	Std. Error	Beta	**t**	p
(Constant)	24.20	100.74		.24	**0.008**
Gender	31.14	13.43	0.65	2.31	**0.03**
Age	-.44	1.43	-0.10	-0.31	0.76
Marital status	-8.51	3.74	0.53	-2.27	**0.03**
No of children	-6.08	5.56	-0.50	-1.09	0.28
Religion	-6.51	4.25	-0.35	-1.53	0.14
Educational level	13.80	10.49	0.32	1.31	0.20
Studying for higher degree	7.07	17.51	0.13	0.40	0.69
Level of study	6.25	17.99	0.12	0.34	0.73
Household size	1.10	2.18	0.14	0.50	0.62
Working experience	2.66	1.02	0.82	2.60	**0.01**
Living in job area	-34.15	11.17	-0.72	-3.05	**0.007**
Rural-urban background	-3.44	9.02	-0.08	-0.38	0.70
Place of residence	1.22	4.88	0.06	0.25	0.80
Job designation	-3.33	3.11	-0.28	-1.07	0.29
No of communities covered	0.92	1.54	0.16	0.59	0.55
No of farmers groups covered	1.06	0.75	0.36	1.40	0.17
No of farmers covered	-3.4E-02	0.01	0.57	-2.00	**0.01**
Means of mobility	1.13	13.15	0.02	.087	0.93
Distance to farmers	-4.0E-02	0.03	0.39	-2.11	**0.02**
Information sources	-.34	0.51	-0.18	-.671	0.51
R	0.84				
R square	0.71				
Adjusted R	0.35				
F	2.98				
P	0.008				

4. Discussions

From Table 1 most of the respondents (82.5%) were males, between 40 to 45 years, 85% were married and 87.5% had Diploma as educational qualification. The trend of the results agrees with Saito, and Weidemen (1990) that extension profession is male dominated in Africa and officers have level low of educational. Also, 54% had between 4 to 6 persons as household size, while 75% were Christians. The most prominent category of extension officers was agricultural technician, and 92.5% had working experience of at least 10 years. Majority of the extension officers live in their job area (82.5), while 80% covered at least 3 communities and 3 farmers group. In terms of number of farmers covered, only 40%of the extension officer covered more than 500 farmers and 45% travelled more than 40 km to reach their farmers.

In Table 2, the result revealed that extension officers were only satisfied with 4 out of 34 indicators of job satisfaction. These were research policies (3.77) work exposure (3.37) identifying farmers' problems (3.02) and opportunities to advance education (3.00). Oladele (2004), Banmeke and Ajayi (2005),and Akinsorotan (2007) reported that in, Nigeria, determinants of extension agents' job satisfaction included ability to identify farmers' problems, opportunity to further education and work exposure. However, extension officers were very dissatisfied with availability of experimental land (1.90) rewarding system (1.97), budgeting (1.90) and sanctions (1.90). The high number of dissatisfying items on the scale is an indicator that there is need for intervention to improve the satisfaction of extension officers in order to stimulate improved performance as expected in the new ERP.

From Table 3, the independent variables were significantly related to job satisfaction with F value of 2.98, $p < 0.05$. Also, R value of 0.84 showed that there was a strong correlation between independent variables and job satisfaction. The result further predicted 71 percent of the variation in job satisfaction by extension officers. Significant determinants were gender ($t = 2.31$), marital status ($t = -2.27$), working experience ($t = 2.60$), living in job area ($t = -3.05$), number of farmers covered ($t = 2.00$) and distance to farmers ($t = -2.11$). It implies that as there are more male extension officers, with long years of working experience their job satisfaction would increase. However as more extension officers are married, do not live in their job area and number and distance to farmers covered increases job satisfaction would decrease.

There was a high level of job dissatisfaction among extension officers in the study area. The items on the dissatisfaction list are areas requiring policy intervention for the improvement of extension delivery to farmers in the study area. There was also the dominance of married males in the extension delivery profession. The education al level of most extension officers was low. Prominent areas of satisfaction among extension officers were research policies, work exposure, identifying farmers' problems and opportunities to advance education. Significant determinants of job satisfaction were gender, marital status, working experience, living in job area, number of farmers covered and distance to farmers. The study therefore recommend that policy makers and extension managers should pay attention to the items indicated for satisfaction and dissatisfaction by the extension officers with a view of boosting their morale for a higher level of performance.

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Skin abnormalities，female reproductive disorders and shorter life span with a mutation in the hairless gene

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Abstract: A spontaneous recessive mutation named rhinocerotic and short-lived（symbol：*hr*^{rhsl}） arose in a breeding colony of Chinese Kunming mice.Mutant *hr* mouse strains show skin and hair abnormalities and shorter life span.The present study analysed the skin，thymus and ovary of young（2 mo）and adult（6 mo）wild type and mutant mice.The mutant mice showed the disintegration of hair follicles and formation of utriculi and dermal cystic structures in the dermis by histology and electron microscopy.The thymus of mutant mice underwent the accelerated atrophy and the decreased number of $CD4^+CD8^-$ and $CD8^+CD4^-$ were examined，and the increased apoptosis in the ovarian granulosa cells were observed in the mutant mice compared with the age-matched wild type by hematoxylin-eosin staining and flow cytometry.Taken together，present results strongly suggest accelerated age-dependent regression of thymus and increased apoptotic cells of ovary in mutant mice compared with the age-matched wild type，which could explain at least in part the immunodeficiency，shorter life span and reproductive disorder. [Life Science Journal 2010;7(3):105-111]. (ISSN: 1097-8135).

Key words: hairless mouse, ovary, thymus, hair follicle, skin

1. Introduction

The hairless gene（*hr*）is one of the molecules that regulates the hair follicle cycling and is found to be associated with a recessively inherited complete loss of body hairs（Panteleyev et al,1999）.A mutation in the *hr*（hairless）gene is responsible for the typical cutaneous phenotype of hairless mice (Panteleyev et al,1998c).The first mutation，*hr*，was collected in a British aviary in 1926（Brooke,1926）.After this，a number of mutation causing generalized hair loss occurred in the laboratory.The most widely studies include the hairless（*hr*），rhino（*hr*^{rh}）and nude（*Hfh1*^{nu}）mutations.The *hr* mutation was found to be autosomal recessive allelic pattern,and mapped to mouse Chromosome 14（Sundberg,1994）.Several such mutations have prove to be homologous to specific human disease and represent useful animal models，for example，melanoma，interleukin-12-deficient，and asthma.Hairless mouse was first proposed as a mouse model for human popular atrichia in 1989（Sundberg,1989）.Mice carrying a mutation in *hr*,or in one of its alleles，typically show immune defects，as well as an aged-related immunodeficiency，consistently these animals are more sensitive to chemical carcinogens and UV-induced skin neoplasma.Moreover，these mutants have a decreased antibody response to thymus-dependent antigens with increasing age.Taken together,these findings indicate a thymic defect（Kawajih et al,1980；Shultzld, 1987）.The long duration of the estrous cycle，the absence of the ovulated oocytes was observed in histopathological studies of estrous cycles and ovaries in mutant B10-*hr*^{rhY} mice strain.The absence of mutation division，ovulation and corpora luteum in ovaries was found in contrast to normal isogenic B10 females（Ignatieva E L et al,1988）.

Several mice lacking hair arose spontaneously in a closed colony of Chinese Kunming mice in our lab，Which was isolated and established a mutant mouse strain termed “Yuyi hairless mice（YYHL）”.The inheritance mode of this phenotype was revealed，by testcross，to have resulted from an autosomal recessive mutation（Zhang et al,2002）. In homozygous YYHL mice，the first hair coat grows normally for the first 12 days after birth.Progressive hair loss was initiated around the eyes，and progresses caudally，resulting in a completely hairless condition within 2 wk with the exception of the vibrissae.As affected mice aged，the skin becomes progressively thickened，loose and redundant as schematically shown Figure 1（Zhang et al,2005）.Of interest，the life span in YYHL mice significantly decreased in contrast to normal Chinese Kunming mice and the hairless homozygous females have reduced reproductive capabilities（Li et al,2002;Du et al,2003）.The present study was designed to analyse the changes in the morphology of mutant mouse skin，thymus and ovary，and thymocytes subpopulation and ovarian granulosa cell were analysed by flow cytometer in mutant mice，which were compared with wild type mice.

2. Materials and methods

Animals and tissues

The skin, thymus and ovary of YYHL homozygous mice aged 2（n=5）and 6（n=5）mo，and of age-matched wild type mice (n=5 and n=5，respectively) were used in this study.Mutant mice strains and normal littermates were obtained from the colony of the Laboratory Animal Center of Zhengzhou University. All mice were cared for according to the Guide for the Care and Use of Laboratory Animals，and the study was approved by the Medical Ethical Committee of Zhengzhou University. The animals were killed by decapitation after deep ether anaesthesia，and the skin from the upper back，thymus and ovary quickly removed.

Histology and transmission electron microscopy

The dorsal skin were removed immediately and biopsies were taken both for TEM and histology

studies.For TEM， samples were washed in 5% sucrose cacodylate buffer， postfixed with 1% osmium tetroxide， dehydrated and embedded in epoxy resin after 4h in Karnovsky's fixative.Ultrathin sections were cut, collected on formvar coated grids and examined in an electron microscope.For histology， the skin， thymus and ovary were fixed in 10% neutral buffered formalin overnight， embedded in paraffin， sectioned at 5 microns,and stained with hematoxylin and eosin.

Thymocytes were prepared from mice (2-mo and 6-mo old) and stained with both FITC-conjugated mouse-specific CD4 and PE-conjugated mouse-specific CD8 monoclonal antibodies for two-color staining. Analysis was performed with a FACScan and the Cell Quest program.

Ovaries from mice were harvested after cervical dislocation and immediately placed in ice-cold saline. The granulose cells of follicle were separated using the method described earlier（Gilbert，1977）.The harvested cells were washed in PBS twice and then incubated with 200 μL RNase A (100μg/mL) at 37°C for 30 min. The cells were stained with Propidium Iodide at 4 °C for 30 min and immediately analyzed by flow cytometry.

Statistical analysis

The data obtained from each mouse was averaged per group and standard deviation of the mean values was calculated. Mean values were compared by nonparametric Mann–Whitney test using SPSS 10 for Windows software package. The results were expressed as the mean value ± SD， and differences at $p < 0.05$ were accepted as the level of significance.

3. Result

Histology

YYHL skin displayed obvious differences to the skin of wide type mouse.The histopathological feature of the YYHL mice was the disintegration of hair follicles and formation of utriculi and dermal cystic structures in the dermis and the dermal cysts grew progressively larger with increasing age， example are showed at 2 month and at 6 months （figure 2）， which was believed to be the main pathomechanism of skin wrinkling， folding and thickening in YYHL mice.

The absolute weight and the growth index between the thymus of wild-type mice and the age-matched YYHL mice were significantly reduced. Histological examination showed cortical atrophy， which had a marked disappearance of lymphocytes from the thymic cortex in the thymus of YYHL mice when compared with the wild-type mouse of the same age.Interestingly, the alterations increased in an age-dependent manner. Morphologically, the granulosa cells in ovarian tissue of YYHL mice demonstrated marked apoptosis characterized by cell shrinkage, membrane blebbing and nuclear condensation， some of which were lost from the granulosa cell layer when compared with the wild type mice.

Skin Ultrastructure

By electron microscopy， the number of cells had increased in the epithelial islets by 14 days after birth, and a centripetal arrangement was seen.In the central part of the cysts， a fibrillar material was observed suggesting degeneration of the central cells.The cysts progressively enlarged and a central cavity had come evident by the age of 28 days（Figure 3）.

Change of the weights and growth index, histopathology and lymphocyte subpopulations in the thymus

As can be seen in Table 1， the weights and relative weights of thymus decreased significantly， and histological examination revealed a marked disappearance of lymphocytes from the thymic cortex in 6 mo YYHL mice as compared to 2 mo YYHL mice and the wild type mice.

The distribution of T-cell subsets in the thymus was analyzed by flow cytometry.In 6 mo YYHL mice, the percentage of $CD4^+CD8^-$ and $CD8^+CD4^-$ thymocytes was lower and the ratio between $CD4^+CD8^-$ and $CD8^+CD4^-$ cells increased as compared to the wide-type mice of the same age. The percentages of $CD8^+CD4^-$ and $CD4^+CD8^-$ thymocytes also decreased both 2 and 6 mo YYHL mice.There was no difference in the percentage of $CD4^+CD8^-$ and $CD8^+CD4^-$ between 2 and 6 mo wide-type mice（Figure 4）.

Figure 1. Appearance of YYHL mutant mice.A： The 2-month-old YYHL mouse on the left demonstrates the characteristic hair loss with loose and redundant skin.B： The 6-month-old YYHL mouse with severe thickening and wrinkling of the skin.

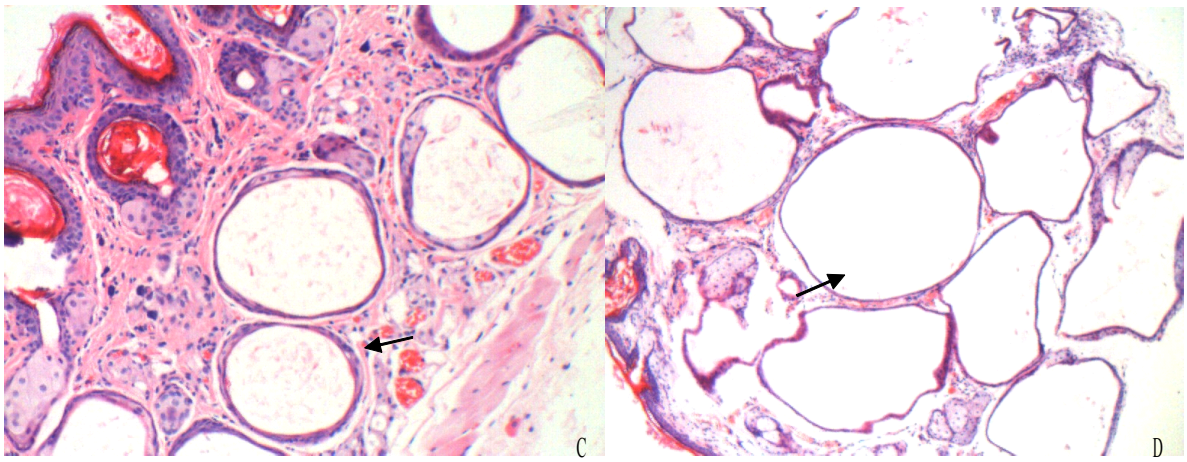

Figure 2. Progressive degeneration of hair follicles in Cross-section of dorsal skin of mutant mice.C： mutant mice at 2 months of age；D：mutant mice at 6 months of age.Solid arrow，enclosed dermal cysts.

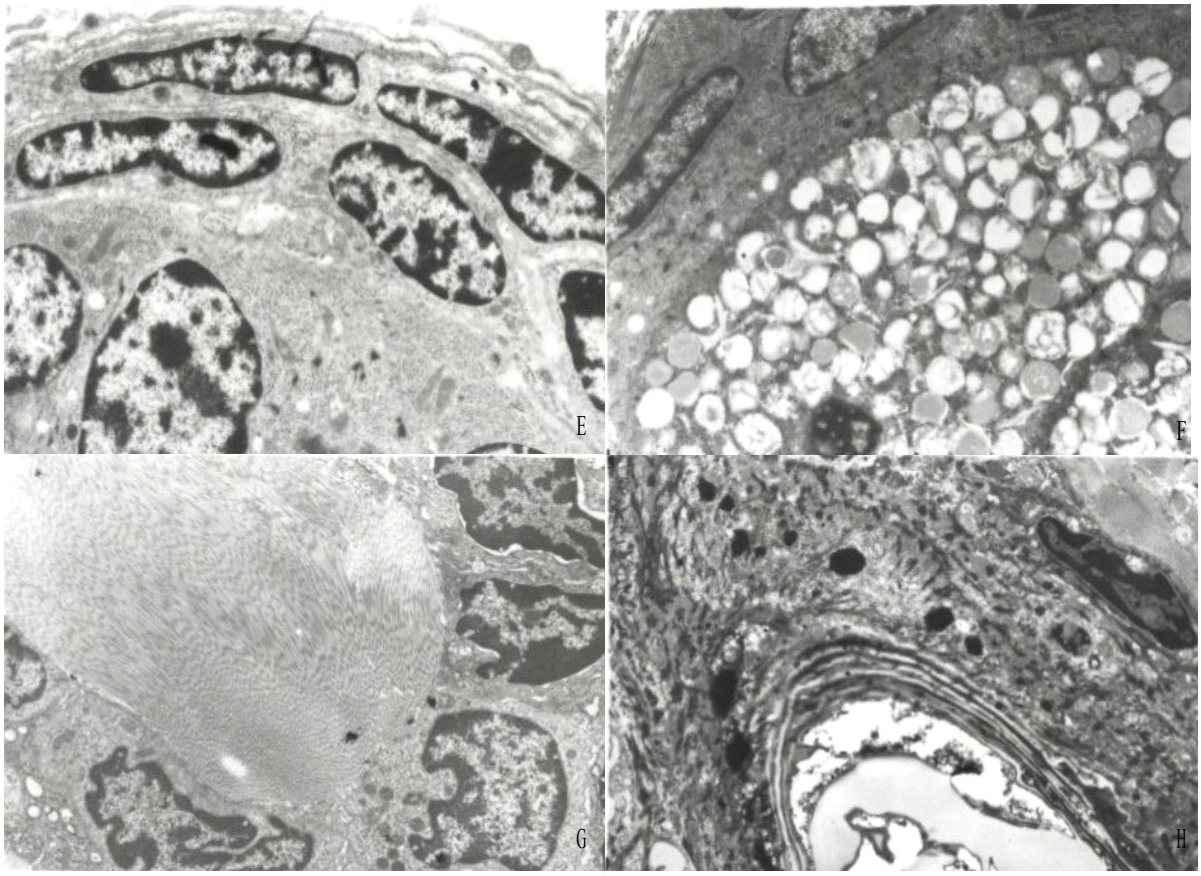

Figure 3. Ultrastructural aspects of dermal cysts. At 12 days（E），dermal cysts are formed by epithelial cells which cluster in islets.Sebocyte-like cells in the cysts of a 16-day-old YYHL mouse（F）.At 20 days（G），the central part of the cyst is characterized by an accumulation of fibrillar material from degenerative cells. At 28 days（H）， The central cavity is formed from degenerative cells.

Table 1. Weight and growth index and lymphocytes subpopulations in the thymus of wild type and mutant mice

	Wild type mice		rhsl-hr-mice	
	2mo	6mo	2mo	6mo
Thymus weight	110.20±7.51	72.50±4.63	85.30±4.65	33.80±1.64[a]
Thymus weight index mg/g body weight（%）	3.18±0.32	1.75±0.09[b]	2.96±0.22	0.74±0.05[a]
$CD4^+CD8^-$	14.33±1.59	13.83±1.63	14.02±1.27	8.85±0.83[a]
$CD8^+CD4^-$	7.81±0.65	8.04±1.12	8.22±1.03	4.22±0.51[a]
$CD4^+CD8^-$/ $CD8^+CD4^-$	1.84±0.25	1.74±0.29	1.71±0.14	2.13±0.41[c]

a，＜0.01 vs all the other groups；b,＜0.05 vs young wild type and young mutated mice；c，＜0.05 vs young wild type,young mutated mice and adult mutated mice.

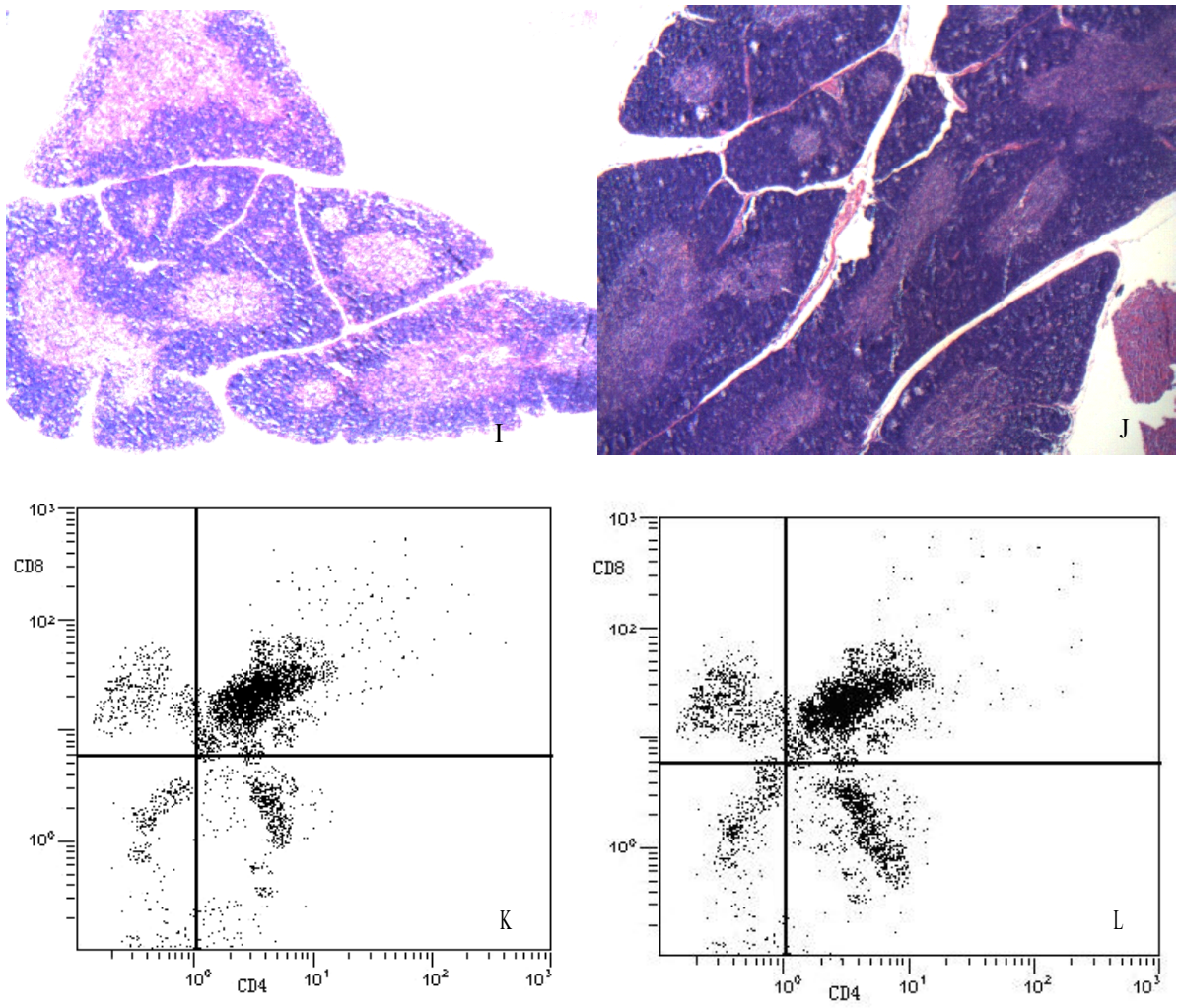

Figure 4. Thymus from 6-month-old mutant and wild type mice. I： Severe thymus atrophy from a mutant mouse showing substantial reductions in the thickness of the cortex and depletion of cortical lymphocytes. J： Normal appearance of the thymus from a wild type mouse showing a thick zone of densely staining cortex and a narrow zone of lightly staining medulla. HE (bar=100μm).Representative flow cytometry profiles of CD4 and CD8 expression on thymocytes both 6 mo YYHL mice （K） and 6 mo wild type mice（L）.Subset percentages were indicated in Table 1.

Analysis of cell cycle and apoptosis on the ovarian granulosa cells

The flow cytometry analyzed that 70.34% and 44.96% of the ovarian granulosa cells in 6 and 2 mo mutant mice respectively were in the G1 phase，which were 28.93% and 32.91% in age-matched wild type mice respectively. 54.89% of the cells in the S phase in 6-mo wild type mice，17.45% in the S phase in 6-mo mutant mice，indicating the accumulation of cells in the G1 phase of cell growth in 6-mo mutant mice as compared to the wild type mice. In the cells undergoing apoptosis，DNA was degraded to fragments of low molecular weight and subsequently leaked out from the cells，and when the DNA content was stained with a DNA-specific fluorochrome，propidium iodide (PI)，a special DNA peak (usually called sub-G1 peak) appeared. Significant apoptosis was present in ovarian granulosa cells of YYHL mice （23.93%） at 6 months compared with YYHL mice at 2 months（7.40%）and 2 and 6 mo wild type mice，which were 3.06% and 2.84% respectively（Table 2）.Histologically，Ovarian granulosa cells demonstrated marked apoptosis characterized by cell shrinkage，membrane blebbing，and nuclear condensation（Figure 5）.The subG1 population and the apoptotic fraction of ovarian granulosa cells was increased significantly in 6 mo mutant mice compared with 2 mo mutant mice and wild type mice（Figure 5）.

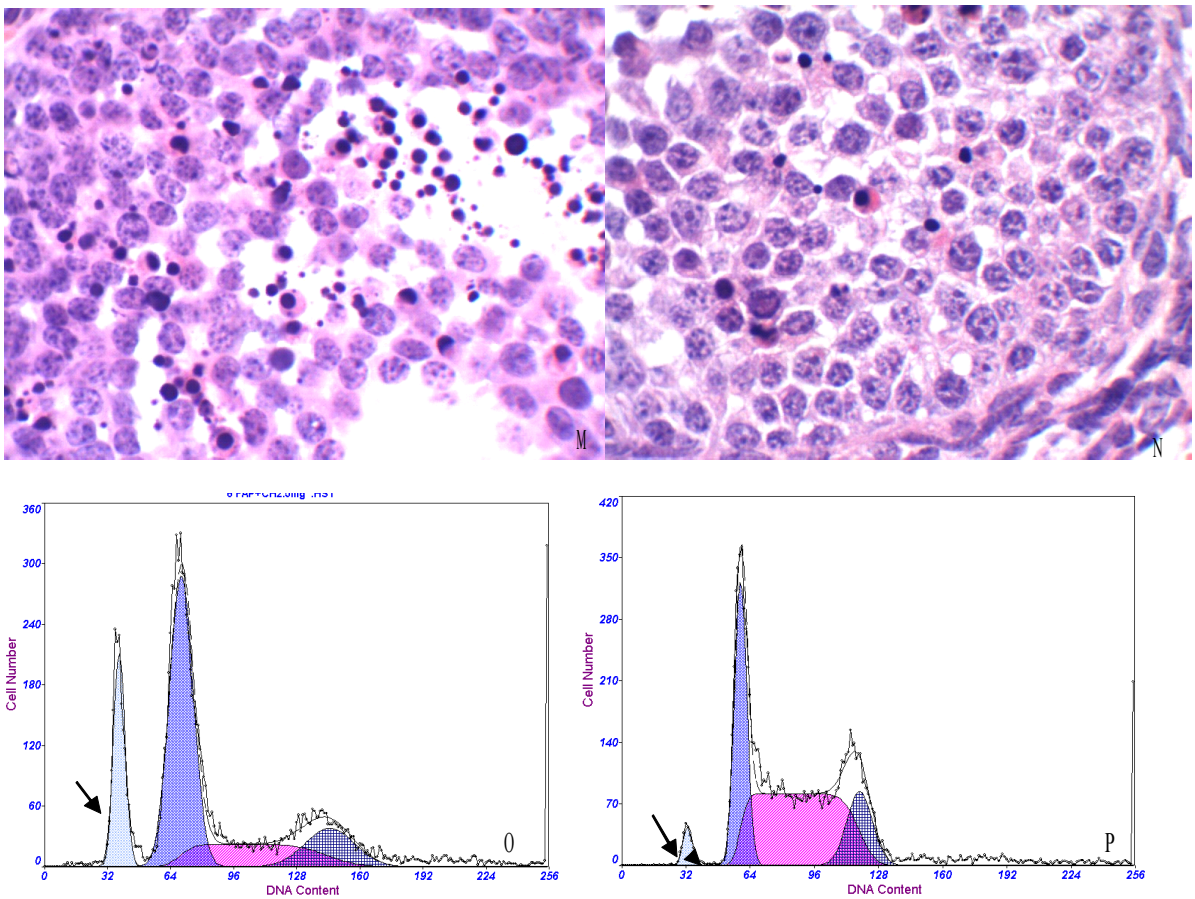

Figure 5. Ovarian granulosa cells were analysed by histology and flow cytometry in the ovaries both mutant mice and wild type mice.Ovarian granlosa cells demonstrated marked apoptosis characterized by cell shrinkage,membrane blebbing， and nuclear condensation in 6 mo YYHL mice （M） compared with the 2 mo YYHL mice and 2 and 6 mo wild type mice（N）.The subG1 population，the apoptotic fraction of ovarian granulosa cells (solid arrow) was increased significantly in 6 mo YYHL mice（O）compared with the 2 mo YYHL mice and 2 and 6 mo wild type mice（P）.

Table 2. Cell cycle and and apoptosis of ovarian granulosa cells in wild type and mutant mice

	Wild type mice		rhsl-hr-mice	
	2mo	6mo	2mo	6mo
G1	32.91±3.86	28.93±2.18	44.96±3.91[b]	70.34±3.70[a]
S	56.24±2.90	54.89±3.24	45.78±2.62[b]	17.45±2.53[a]
G2/M	10.85±2.04	16.18±2.77	9.26±1.01[c]	12.21±2.14
Apoptotic（%）	3.06±0.51	2.84±0.39	7.40±1.20[b]	23.93±2.35[a]

a，＜0.01 vs all the other groups；b,＜0.01 vs young wild type mice and wild type mice；c,＜0.01 vs adult wild type mice and adult mutated mice,young mutated mice and adult mutated mice.

4. Discussion

In this study， we reported that a newly found mutant mouse（YYHL）has histological and Ultrastructural features in the skin.Histologically，the main reason for the abnormal skin was the various sizes of dermal cysts occupied the lower dermis and subcutaneous tissue.It was worth noting that dermal cysts in the skin of aged hairless mice underwent a slow enlargement process， which resulted in the abnormality of skin.The TEM study showed the characteristics of cysts that developed in the deep dermis of hairless mice.Early development of dermal cysts was primarily characterized by an increase in the number of cells in the islets.Simultaneously the central part of the cyst was characterized by an accumulation of fibrillar material from degenerative cells. At later stages of development，a central cavity formed which derived from degeneration of the central cells.Our results ruled out the possibility that these cells might correspond to cells originating from the follicular dermal papilla which are mesenchymal cells （Roth,1965;Montagna,1974） .The study analyzed the thymus of 2 mo and 6 mo mice carrying a mutation in the *hr* gene.Here we described for the first time structural changes as well as T-cell subsets of thymus in YYHL mice .The result revealed the reduction both the weight and the weight index of the thymus in 6 mo YYHL mice compared with the wide type mice of the same age.The main structural change in the thymus of mutated mice was the atrophy， which showed substantial reduction in the thickness of the cortex and the depletion of cortical lymphocytes.A previous study reported atrophy

of the thymus of hr-rh-j mice with aging（San Jose et al，2001）.In the thymus，functionally mature SP thymocytes derive from immature DP cells through negative and positive selections (Penit and Vasseur，1989； Nossal，1994) mediated by complex cellular interactions， and a variety of cytokines， hormones， neuropeptides， and growth factors (Takayama et al， 1998). In turn， DP thymocytes derive from immature DN cells， and then express the TCRb polypeptide chain on their membrane (Wurch et al，1999).Our results showed that a decrease in $CD4^+CD8^-$ and $CD8^+CD4^-$ thymocytes and the ratio between $CD8^+CD4^-$ and $CD4^+CD8^-$ in 6 mo YYHL mice compared with the wide-type mice of the same age. Several of the mouse mutations with abnormalities of the integument associated with more or less severe immunological defects have been reported previously（Shultz LD et al,1978）.Our findings suggested an age-dependent degeneration and accelerated regression of the thymus in YYHL mice，which might be correlated with the impaired function of cellular immunity and shorten life span.

In this study，we examined the apoptosis and the cell cycle of ovarian granulosa cell both mutanted mice and wild type mice by flow cytometry.The present results showed that a G1-phase to S-phase cell cycle block was accompanied by increased apoptosis cells in 6 mo mutated mice compared with 2 mo mutated mice and young and adult wide type mice. In addition， histological analysis also demonstrated the increased granulosa cell apoptosis by hematoxylin-eosin staining and the presence of apoptosis was associated with decreased ovarian weight in mutant mice.Previous studies describing the reproductive defects in hr^{rhY}/hr^{rhY} mice. It was proposed that the reason for hr^{rhY}/hr^{rhY} female infertility was not the dysfunction of the ovary itself， but the impaired perception of any external regulatory factors， most likely， luteinizing hormone of the anterior pituitary， which played an important role in the regulation of ovulation（Garcia and Jones,1981 ） .Our study revealed it was possible that mutation induced apoptosis of ovarian granulosa cell caused reproductive disorders in Yuyi hairless female mice.

Taken together， our findings strongly suggest accelerated age-dependent regression of thymus and increased apoptotic cells of ovary in mutant mice compare with the age-matched wild type， which could explain at least in part the immunodeficiency，shorter life span both YYHL male and female mice ， and the reduced reproductive capabilities in YYHL female mice.More studies are necessary in order to elucidate the true origin of these in mutated mice.

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9/16/2010

Subconjunctival bevacizumab, a potential therapeutic strategy for treatment of corneal neovascularization.

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Abstract: Purpose: feasibility of local application of bevacizumab for inhibition and treatment of corneal angiogenesis. Materials and Methods: 20 pigmented rabbits with average weight 3.7±0.4 kg were numbered and two groups were made, Group A: the rabbits of this group were subjected to corneal sutures application to induce corneal vascularization. Group B: rabbits of this group were subjected to corneal sutures with concomitant bevacizumab application. The rabbits were kept under observation and were examined and photographed after one week of taking the deep corneal suture for assessment of the corneal vessels. Then rabbits of group B were anaesthetized and bevacizumab was injected. At the end of the experiment, the rabbits were killed with an intravenous overdose of thiopentone, and histopathological studies were done. All histopathological analyses were performed by investigators blinded to medicine injected and the group of bevacizumab injection. Results: Corneal neovascularization disappeared in all rabbits of the group of bevacizumab injection. Conclusion: Data presented in this study effectively demonstrates the potential feasibility and safety of local application of bevacizumab for inhibition and treatment of corneal angiogenesis in an animal model. [Life Science Journal 2010;7(3):112-116]. (ISSN: 1097-8135).

Keywords: Subconjunctival, bevacizumab, corneal neovascularization.

1. Introduction

Neovascular diseases of the cornea and other parts of the eye represent a major public health problem. A wide range of inflammatory, infectious, degenerative, or traumatic disorders may induce corneal neovascularization. (1) Corneal neovascularization is a major challenge following chemical burns and corneal inflammation. Corneal neovascularization is a sequela of several inflammatory diseases of the anterior segment, such as infections, reactions to corneal transplantation and extended contact lens wear. The potential of antiangiogenic therapy has been greeted with great hope in ophthalmology. The tight regulation of corneal neovascularization helps maintain the transparency and immune privilege of the cornea. (2)

Corneal avascularity requires low levels of angiogenic factors and high levels of anti-angiogenic factors under basal conditions. Rupture of this homeostasis may occur in the pathogenesis of corneal neovascularization.(1) The data supporting a causal role for VEGF in corneal neovascularization are extensive. (3 - 13)

During corneal neovascularization, an up-regulation of angiogenic factors must be present, most likely in association with a down-regulation of anti-angiogenic molecules. It was recently shown that VEGF was up-regulated in inflamed and vascularised corneas in humans and in animal models. (11, 12, and 14) Angiogenesis is controlled by several mediators, including VEGF and basic fibroblast growth factor.

VEGF promotes several steps of angiogenesis, including proteolytic activities (dissolution of the membrane of the original vessel), endothelial cell proliferation, migration, and capillary tube formation. (1)

Hypothesis

Taken together, these data indicate that an anti-VEGF therapeutic approach may limit the visual loss associated with conjunctivalization of the corneal surface. Therefore, we propose the hypothesis that local administration of new anti-VEGF compounds such as pegaptanib sodium, bevacizumab and ranibizumab may be safe and an effective therapeutic option in corneal neovascularization.

To test this hypothesis the effects of a subconjunctival injection of bevacizumab (Avastin) tested in an animal model of corneal neovascularization.

2. Material and Methods

This protocol was approved by the Research Institute of Ophthalmology Medical Committee and was conducted in accordance with regulatory guidelines for the care of laboratory animals. We used 20 rabbits with average weight 3.7±0.4 kg.

All rabbits were examined by slit lamp to exclude any eye pathology or corneal neovascularization.

Deep corneo-limbal stitch were done in all rabbits to induce corneal neovascularization, the rabbits were anaesthetized and 8 0 vicryl sutures were taken under surgical microscope.

All rabbits were examined and photographed after one week of the application of the deep corneo-limbal suture, and the area of neovascularization was assessed according to the size (objectively the area of neovascularization was measured by ruler) and density (subjectively by the examiner)

The rabbits were numbered and two groups were made each contain 10 rabits:

Group A: rabbits of this group were taken as control, saline (2, 5 mg) were injected sub-conjunctively near the area of neovascularization.

Group B: rabbits of this group were injected by bevacizumab (2, 5 mg 0,1ml.) sub-conjunctively near the area of neovascularization.

Then rabbits of both groups were anaesthetized and saline and bevacizumab was injected (1 week after corneal sutures) in each group. For local delivery of saline & bevacizumab we used the insulin syringe for subconjunctival injection near the area of neovascularization.

Rabbits in the 2 groups were kept under observation and were re-examined and photographed after one week of injections, (2 weeks after deep corneal sutures) for assessment of the area of neovascularization (both objectively and subjectively) and grading scale were fashioned.

Table 1. Change in size of area of neovascularization

Grade	Change in size of area of neovascularization
0	No change
I	25 % Reduction
II	50 % Reduction
III	75 % Reduction
IV	Complete resolution

At the end of the experiment, the rabbits were killed with an intravenous overdose of thiopentone, the eye were enucleated and immediately bisected and fixed in 2, 5 % buffered glutaraldehyde for 6 hours, then sections from the cornea were taken, washed in phosphate buffer, post fixed in 1 % osmium tetroxide, dehydrated in a series of graded ethanol and lastly embedded in Araldite cy212. Semi thin sections of one micron thickness were obtained and stained with Toluidine blue (TB) stain for light microscopic examination.

All histological analyses were performed by investigators blinded to medicine injected and the group of bevacizumab injection.

3. Results and Discussion

In all 20 rabbits, the deep corneal sutures induced corneal vascularization (Fig. 1-2); the average area of neovascularization was 2 x 1 mm. (Table 1).

The results after the injections are summarized in Table 2.

In animals of **group A**; the area of neovascularization did not regress after saline injection, no change in the size or density occurred. (Table 2)

In **group B**; the area of neovascularization was considerably reduced both in size and density in all rabbits. Disappearance of the corneal neovascularization occurred in 7 rabbits (70 %). (Fig. 3) In 2 of injected rabbits (20 %), the area of neovascularization were reduced by more than 75 % in size with marked reduction in density and caliber of the blood vessels, in 1 of the injected rabbits (10 %) the area of neovascularization reduced to more than 50 %, with marked reduction in density of the neo-vessels. (Table 2).

Histopathologic Assessment

Light microscopic examination of the prepared corneal sections in the current stud included the following:

Group A: rabbits of this group were subjected to corneal sutures application to induce corneal vascularization with saline injection. Microscopic examination of corneal sections revealed multiple vascular spaces with disarranged surrounding collageneous lamellae (Fig 4, 5).

Group B: rabbits of this group were subjected to corneal sutures with concomitant bevacizumab application Microscopic examination showed different stages of occlusion of the induced neovascularization. This was in the form of narrowing, partial obliteration and subsequent complete occlusion of the luminae (Fig 5, 6, 7, 8, 9)

Table 2. Clinical evaluation of the 2 groups

	No. of rabbits	Area of neovascularization 1 W after suture	Size 1 W after injection	Grade	Percentage Of rabbits improved
Group A	10	2 X 1 mm	2 X 1 mm	0	0 %
Group B	7	2 X 1 mm	Complete resolution	IV	70 %
	2	2 X 1 mm	Reduction in size more than 75 %	III	20 %
	1	2 X 1 mm	Reduction in size more than 50 %	II	10 %

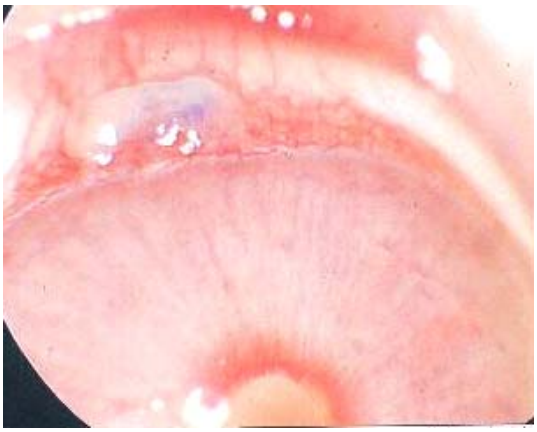

Fig. (1)

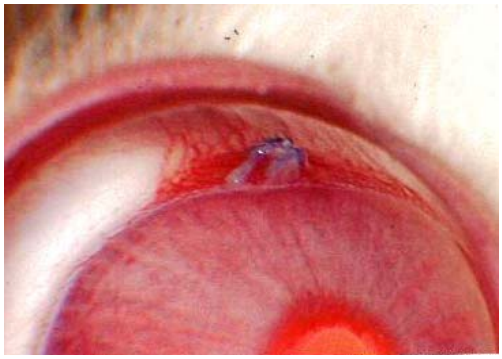

Fig. (2)

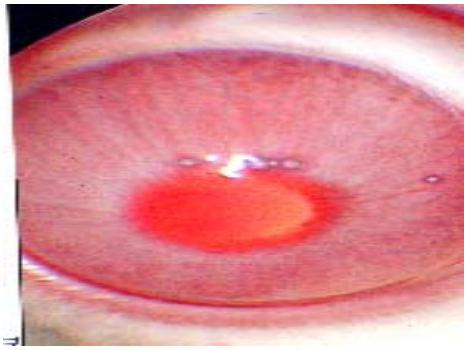

Fig. (3)

Fig. (4)

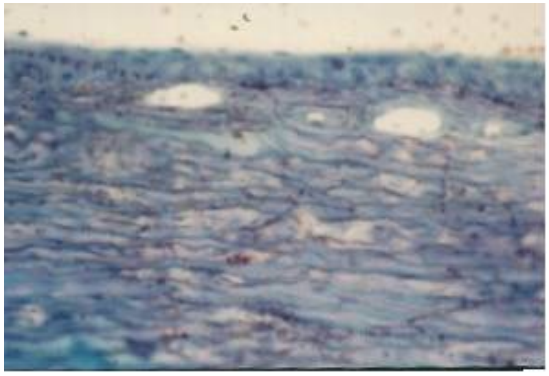

Fig. (5)

Fig. (6)

Fig. (7)

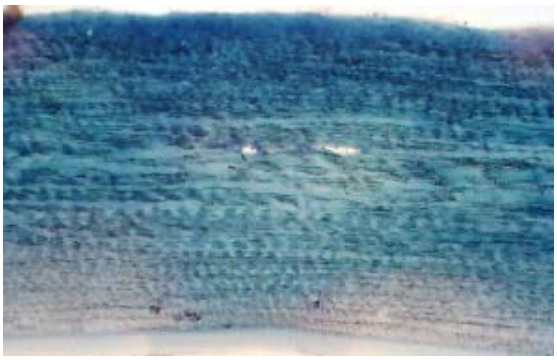

Fig. (8)

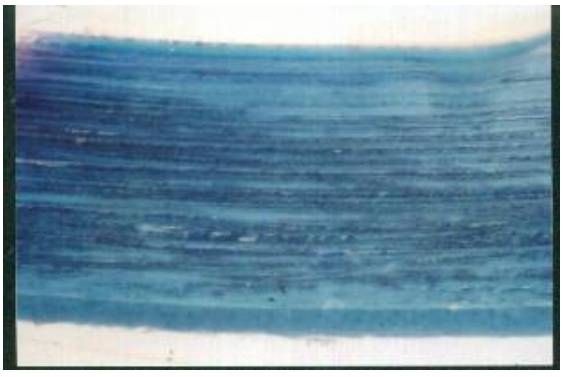

Fig. (9)

4. Discussion

Corneal neovascularization is a serious complication that can occur following keratoplasty and predispose to graft rejection. It is characterized by the growth of new capillaries from the limbal blood vessels and is often accompanied by an inflammatory response. The neovascularization starts in the recipient corneal bed, then extends to the graft–host interface and finally to the graft itself. Corneal neovascularization is a high risk for graft rejection after corneal transplantation because of the associated high levels of inflammatory cells and inflammatory mediators within the graft, which eventually provoke rejection and failure. (25).

Prevention and control of corneal neovascularization is thus a critical step in stopping graft rejection and failure.

Corneal neovascularization appears to be controlled by 2 opposing mechanisms: angiogenic stimulators such as vascular endothelial growth factor (VEGF) and angiogenic inhibitors such as angiostatin. (15, 16, 17).

Under normal conditions, the balance is toward the endogenous angiogenic inhibitors, keeping the cornea avascular. An insult to the cornea, may enhance the production of angiogenic stimulators, disturbing the balance and resulting in capillary endothelial cell proliferation and neovascularization.(15).

Vascular endothelial growth factor is a potent and highly selective mitogen for vascular endothelial cells, as well as a modulator of vascular permeability (vascular permeability factor), and is thought to play a key role in the pathogenesis of corneal neovessels. (20, 21)

Several methods to treat corneal neovascularization have been tried, argon laser photocoagulation and photodynamic therapy with different photosensitizers. However, such therapies are associated with thermal damage to the cornea and a high rate of vessel recanalization. Pharmacological treatment of corneal neovascularization using angiogenic inhibitors has evolved as a new way to manage corneal neovascularization. Lately, the success of monoclonal antibodies against VEGF such as bevacizumab and ranibizumab (Lucentis) in the treatment of retinal and choroidal neovascularization has encouraged the use of these antibodies to treat corneal neovascularization. (22, 23)

An important question is how to achieve therapeutic concentrations of such antibodies in the cornea.

Because there is little knowledge of the rate of absorption of the drug through the subconjunctival route and the required therapeutic dose, the answer to this question is uncertain. For most drugs, the recommended subconjunctival dose is 10 to 100 times the intravitreal dose. It seems logical that for control of corneal neovascularization, higher doses of bevacizumab than the classic 2.5 mg given in intravitreal injections are needed for subconjunctival injections (repeat injections may also be needed).

However, further studies are needed to establish the exact dose needed. This study may point to a possible role for VEGF inhibitors in the management of corneal neovascularization and, possibly, graft rejection. Topical application is another possible route. (24)

5. Conclusion:

Data presented in this study effectively demonstrates the potential feasibility of local application of bevacizumab for inhibition and treatment of corneal angiogenesis in an animal model**.**

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Fig (1-2): Colored photos of induced corneal neovascularization by deep corneo-limbal 8 0 vicryle suture.

Fig (3): Colored photo after I week of bevacizumab injection.

Fig (4): light photomicrograph of semi thin section of control rabbit cornea with induced neovascularization showing an empty oval vascular space (v) inbetween the stromal collageneous lamellae (c) (TB stain X 500).

Fig (5): light photomicrograph of semi thin section of control rabbit cornea with induced neovascularization showing multiple oval to round empty vascular spaces (v) with surrounding disarrayed collageneous lamellae (c) (TB stain X 500).

Fig (6): light photomicrograph of semi thin section of control rabbit cornea with induced neovascularization with bevacizumab application showing partial occlusion of newly formed vessels (v) (TB stain X 250).

Fig (7): light photomicrograph with higher magnification showing partial occlusion of the newly formed vessels (v) (TB stain X 250).

Fig (8): light photomicrograph of semi thin section of control rabbit cornea with induced neovascularization with bevacizumab application showing almost completely occluded slit-like vascular space (arrow) (TB stain X 250).

Fig (9): light photomicrograph of semi thin section of control rabbit cornea with induced neovascularization with bevacizumab application showing evidence of completely occluded vascular space (arrow) (TB stain X 250).

7/7/2010

The Protective Role of Alpha Lipoic Acid Against pesticides Induced testicular toxicity. (Histopathological and Histochemical Studies)

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Abstract: The present study aimed to investigate the efficiency of alpha-lipoic acid (ALA) as natural antioxidant in ameliorating some of changes induced by intoxication with a mixture of well known pesticides used in our agricultural media. Four groups of male rats were treated as follows untreated control animals, (p-mix, consists of 1/60LD50 chloropyrifos (2mg/Kg b.wt) 1/200 LD50 of fenitrothion (2.5 mg/km b.wt) as used in agriculutural environment and ALA 200mg/animal of alpha lipoic acid, (P-mix+ALA). Histological observation of the intoxicated rats revealed significant alterations in the testis tissue of P mix. treated group including focal mild testicular damage, blood hemorrhage and hypospermatogensis, necrosis and atrophy. The degree of fibrosis was encountered using masson-trichrome stain technique which revealed various fibrosis grades between the control and treated testes tissues upon the exposure to the insecticides. TUNEL technique showed an increase in the incidence of positive apoptotic cells between the spermatogonial and germ cells. Also complete depletion of the level of acid phosphatase enzyme which involved in the testosterone biosynthesis. The treatment with alpha lipoic acid showed many degrees of improvements in the seminiferous tubules, spermatogenic germ cells and the interstitial cells. Also decrease in the grades of fibrosis between testes tissues. Conclusion: The biochemical, hiopathological, reports supported that the pesticides have many implicated toxic changes on the testes tissues and the antioxidants like alpha lipoic acid obtained many trials to get ameliorative effects on the toxicity of pesticides. [Life Science Journal 2010;7(3):117-124]. (ISSN: 1097-8135).

Key Words: Pesticides – Reproduction - Apoptosis - Fibrosis – Antioxidants

1. Introduction:

Chlorpyrifos, first introduced into the marketplace in 1965, has been widely used globally as an insecticide to control crop pests in agriculture, reduce household pests such as termites, reduce insect damage, and for mosquito control. Fenitrothion is approved as a broad-spectrum organophosphorus pesticide. Its toxicity was first evaluated by the 1969. Problems associated with pesticides hazards to man and environment are not confined to the developing countries, but extended to developed nations and still facing some problems in certain locations (Nuckols *et al.,* 2007 and Suresh, 2007). It has many structural actions of insecticides as the inhibition of the release of the acetylcholonesterase at the synaptic junction (Roy *et al.,* 2004). Several studies showed that organophosphorous as malathion and chlorpyrifose induced various physiological, biochemical, immunological and histological changes in experimental animals (Tamura *et al.,* 2001 and Selvakumar *et al.,* 2004). The widespread usage of organophosphates has stimulated research to the existence of effects related with their reproductive toxic activity (Pajoumand *et al.* 2002).

Hileman, (1994) reported that fenitrithione have the potential to cause reproductive toxicity in animals, affect human reproduction. Okamura *et al.,* 2005 and Presibella *et al.,* 2005). were showed some pathological effects of pesticides on the reproductive system of experimental animals. Chlorpyrifose had been obtained testicular damage, damage to sperm production, and reduction in testosterone levels when fed to adult male rats (Afifi *et al.,* 1991). There is growing concern that pesticide as chlorpyrifose, had estrogenic property may be causing a variety of reproductive disorders in wildlife and human population (Chitra *et al.,* 1999).

Gangadharan *et al.* (2001) was reported that organophosphorous have been shown to produce reactive oxygen species (ROS) in both intra and extra cellular spaces, resulting in decline of sperm count and infertility in wildlife and human. Chlorpyrifos was showed severe testicular damage and results in reduction in sperm count affecting fertility (Ibrahim and ElGamal, 2003). Chlorpyrifos (CPF) and Fenitrothion, are organophosphorous insecticides, have postulated a possible role for the generation of free radicals and induction of oxidative stress (Tuzmen *et al.,* 2008). Suskind *et al.* (2007) was reported that a significant correlation between increased fibrosis and both reduced tubular diameter and fewer germ cells.

Apoptosis or programmed cell death is an active process controls cell numbers in a variety of tissues and at various phases of germ cell development (Bartke, 1995). Many studies reported that many organophosphorous as chlorpyrifose caused withdrawal of gonadotropins and/or testosterone which enhances the germ cell degeneration and apoptosis of germ cells in the testes (Billig *et al.,* 1995; Henriksen *et al.,* 1995; Hikim *et al.,* 1995; Kangasniemi *et al.,* 1995).

Lipoic acid is an organ sulfur compound, which is an essential cofactor for many enzyme complexes and the amount of lipoic acid present is very low. Naturally occurring lipoic acid is always covalently bound and not immediately available from dietary sources. Studies are generally dealing with the biological consequences of lipoic acid administration and its derivatives in cases associated with oxidative stress (Han *et al.,* 1997 and Henriksen, 2006).

An attempt was made to elucidate the possible protective effect of-lipoic acid treatment on pesticides-induced physiological and histopathological alterations in rats with testicular toxicity. Chlorpyrifos exposed rats showed abnormal levels of antioxidants enzymes In contrast, rats pretreated with lipoic acid showed normal lipid peroxidation and antioxidant defenses. These findings indicate a cytoprotective role of lipoic acid in this experimental model of testicular toxicity (Selvakumar *et al.,* 2004). Present study was taken to assess the effects of chlorpyrifos and fentrithion on testes, the main organ of male reproduction and the possible ameliorative effect of naturally occurring antioxidants like alpha lipoic acid.

2. Materials and methods

Animals and experimental design:

Animals:

Male albino rats *Rattus norvegicus* (3–4) month's age, weighing between 150–180 g were used. Animals were supplied by the breeding unit of the Egyptian Organization for the Biology and Vaccine Production, Egypt. The animals were housed in plastic cages, fed *ad libitum* and allowed to adjust to the new environment for two weeks before starting the experiment. The rats were housed at 23 ± 2 ^{o}C dark/light cycle.

Chemicals:

Chlotpyrifos:

Pyriban (chlorpyrifos 48%EC) (O,O–Diethyl-O(3,5,6-trichloro-2-pyridyl phosphorothioat) was supplied by El Help company for pesticide industry- Egypt.

Fenitrothion:

Sumithion (Fenitrothion 50% EC) (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate) was purchased from Kaffer Elzayat Co. for Insecticide Ind. Kaffr Elzayat, Egypt**.**

Antioxidant used: Alpha lipoic acid

Experimental Design:

All animals were treated according to the standard procedures laid down by OECD guidelines 407 (1992) repeated dose 28 days oral toxicity study in rodents. Animals were randomly divided into six experimental groups, five animals each as follows:

Group I (control): each animal in this group was given distilled water (1ml/animal) by gastric incubation every day for 28 days.

2- Group (P-mix): rats were orally treated via gastric intubation with mixture of pesticides mixture contain (1/60LD_{50} chloropyrifos (2mg/Kg b.wt, 1/200 LD_{50} of fenitrothion =2.5 mg/k gm b.wt every day for 28 consecutive days.

3- Group (ALA): rats were orally supplemented with 60mg /Kg for 28 days and served as +ve control. 4-Group (P-mix + ALA): rats were orally supplemented with (60 mg /kg) ALA 1 hour after intoxication with pesticides mixture.

Sampling

Blood samples collected from the retro-orbital plexus vein according to Schermer (1967). On heparinzed tubes at 28 days of treatment periods, plasma samples were separated by centrifugation of the blood samples at 3600 rpm for 15 min. Plasma samples were kept at -20 C^{o} for subsequent use. At the end of the experiment, animals were dissected and samples of the testes were subjected to the histopathological and histochemical studies.

Biochemical assay

Malondaldehyde (MDA) enzyme was measured according to Ohkawa *et al,* (1979) in the plasma after incubation at 95°C with thiobarbituric acid in aerobic conditions (pH 3.4). Testosterone hormone level was measured in the plasma according to Tremblay, (2001).

Histological studies:

Animals were sacrificed after 24 hour of treatment. The testis was dissected and fixed immediately in neutral buffered formalin (10%) and paraffin sections were prepared and stained with hematoxylin and eosin. and Masson-trichrome stain was used showing collagen and elastic fibers changes according to Bancroft and Stevens, (2002).

2- Assessment of apoptosis.

Evaluation of apoptosis in testis tissue homogenate was achieved by quantification of cytoplasmic histone-associated DNA fragments using cell death Detection ELISA plus kit (Roche). One ml of testis tissue was transferred into 1 volume incubation buffer (7% paraformaldehyde) and homogenized. According to the kit manufacturer's guidelines (Roche), homogenized samples were centrifuged at 13000 rpm for 10 min at 4ºC, the supernatant was removed carefully, and the pellet was resuspended in 200 µl incubation lysis buffer, and incubated for 30 min at room temperature. It should be noticed that several dilutions of testis tissue were assayed to determine the appropriate concentration required for ELISA as a preliminary test. Then the lysate was centrifuged at 200x g for 10 min, the supernatant (cytoplamic fraction) 20µl/well was transferred carefully into the streptavidin-coated micro-titer plate (MTP) for analysis; samples were added in duplicates. Positive, blank and background controls were treated similarly as the samples. The immunoreagent was prepared by mixing 1/20 volume antihistone-biotin with 1/20 volume anti-histone with 18/20 volume incubation buffer (v:v:v), then 80µl/well of the prepared reagent were added to MTP. The plate was incubated (covered with adhesive foil) on MTP shaker under gentle shaking for 2 hrs at room temperature. Then, the solution was well rinsed in 250 µl incubation buffer. The reaction was visualized by adding 100 µl/well of the freshly prepared substrate ABTS, incubated for 15 min on a plate shaker at 250 rpm until the colour development is sufficient for photometric analysis. The absorbance was recorded at 405 nm against ABST as a blank (reference wave length approx. 490 nm). Unless otherwise stated, all reagents and supplements were

supplied with the kit. The concentration of nucleosomes in the sample reflects the amount of cell death. Increases in DNA fragmentations over control values (blank and background) were measured and expressed as $OD_{405-490}$.

TUNEL staining.

To detect cells undergoing apoptosis, the tissue sections were stained according to the TUNEL procedure (Gavrieli *et al.,* 1992), with some modifications. Briefly, the testes tissues was immediately fixed in 4% paraformaldhyde at 4ºC for 20 – 22 h and embedded in paraffin. The tissue was sectioned at 4µm, dewaxed, rehydrated, and digested with 20µg/ml of proteinase K (Sigma). Endogenous peroxidase was blocked by treatment in 0.3 % hydrogen peroxide. The sections were then rinsed in water and incubated with 50µl of terminal deoxynucleotidyl transferase buffer in a moist chamber at 37ºC for 60 min. The sections were then rinsed and 50µl converter-POD was added on each tissue sample, covered, and incubated for 30 min at 37ºC. For colour development the slides were rinsed in PBS, then 50µl DAB-substrate (Roche) solution were added, incubated in dark for 10 min at room temperature, washed, counterstained with haematoxylin, dehydrated and finally coverslips were mounted.

Histochemical study:

The specimens were subjected to the fixation with froml – calcium and acid phosphatase was detected due to Gomori-lead method in which acid phosphatase activity acquire black colour and the nuclei acquired green colours according to Bancroft and Stevens, (2002).

3. Results:

The expressed data in Table (1) declared that in addition to the classical mechanism of pesticides there is an enhancement for the free radicals that expressed by significant elevation in oxidative stress biomarker malondialdehyde (MDA) versus control at $p< 0.05$. On the other hand, consecutive supplementation with ALA for 28 days alone or in combination with pesticides induced observable significant reduction in plasma MDA level, this significant was versus control and P-mix treated groups at $p< 0.05$. As regards to plasma testosterone level repeated intoxication with p mix induced remarkable significant reduction versus control in plasma testosterone level at $p<0.05$. However, supplementation with ALA improves the toxic effect of p mix that was significant versus control and P- mix groups.

Histopathological Observations

(A) Examination of sections of the testes with haematoxylin and eosin stain revealed normal architecture of seminiferous tubules, normal arrangement of spermatogenic cells germ cells and well arranged and distributed interstitial cells in the peritubular areas (Fig1). The second group of rats was treated with (60mg/kgm b.wt.) of alpha-lipoic acid served as positive control showed nearly normal appearance of the seminiferous tubules and Leydig cells (Fig. 2). Upon the toxicity with (P-mix, consists of, 1/60LD50 chloropyrifos (2mg/Kg b.wt)1/200 LD50 of fenitrothion (2.5 mg/kgm b.wt) of pesticides mixture, the testes tissues appeared with many foldings of the basement membrane of the seminiferous tubules, highly degeneration of the interstitial cells (Fig. 3). Also, severe disorganized and atrophy of tubules and complete blood hemorrhage (Fig. 4), some giant and necrotic cells appeared between the interstitial cells (Fig. 5). When the rats treated with alpha-lipoic acid after the toxicity with the pesticides mixtures, the testis tissues revealed some degeneration between the germ cells of the tubules and well arranged interstitial cells (Fig. 6) and well organized.

(B) Fibrosis: Staining of the testes sections with Masson-trichrome stain showed normal thickening of the walls surrounding the testis tissues (Tunica albuginea)(Fig. 8). The testis tissue of the animals treated with (60mg/kg b.wt.) of lipoic acid showed mild fibrosis around the testis section (Fig. 9). The pesticides mixture treatment obtained higher grades of fibrosis surrounding the testis section (tunica albuginea) and in the peritubular areas (Fig. 10, 11). Supplementation of alpha lipoic acid after the toxicity of pesticides mixture showed testis tissues with minimal fibrosis (Fig. 12).

Table (1): Effect of Alpha Lipoic Acid supplementation on Different biochemical parameters in plasma of male albino rats intoxicated with mixture of pesticides(cpf+fn)

Parameter / Groups	Con.	P-Mix	ALA (+ ve C)	ALA+ P-mix
MDA (µmol/dl)	17.22 ± 1.77	31.71 ± 1.66 [a]	19.97 ± 3.57	21.96 ± 2.99
Testosterone (ng/dl)	211.699±20.11	143.043± 11.16 [a]	207.699 ±16.22	192.779 ± 19.27[ab]

All data were expressed as mean ± SE.

[a] significance difference versus control at P<0.05. [b] significance difference versus control at P<0.05.

[c] significance difference versus control at P<0.05. MAD = malondialdehyde

Immunohistochemical results:

Testes sections of all groups were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of tissue sections TUNEL for the detection of apoptosis. The testis tissue of the untreated control rats showed normal significant positive cells (Fig. 13). Upon supplementation with alpha lipoic acid alone the testis tissue revealed mild increase in the positive cells between their germ cells (Fig. 14). The testis section of the animals intoxicated with pesticides mixture obtained significant highly positive cells between all the stages of spermatogenesis cells (Fig. 15). Mild improvement of the apoptotic positive cells was recorded within the testis tissue of the rats treated with alpha lipoic acid after the intoxication with pesticides mixture (Fig. 16).

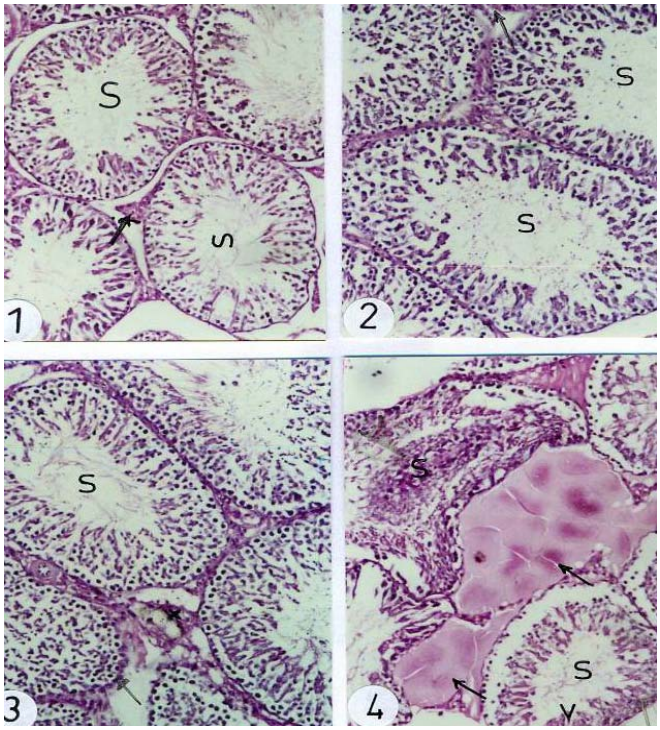

Fig. (1): Photomicrograph of testis section of untreated rat showed normal appearance of testis structure (Arrows) and smeiniferous tubules (S).

Fig. (2) Photomicrograph of testes section of rat treated with (60mg/kg) alpha lipoic acid showed nearly normal architecture of seminiferous tubules and interstitial cells (arrows).

Fig. (3, 4): Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifose and 1/200 LD50 of fenitithione) showed infoldings of the membranes of seminiferous tubules, degeneration of the spermatogenesis process and hemorrhage between the seminiferous tubules (arrows).

Histochemical observations:

Acid phosphatase enzyme was detected in all testes sections of the animals using Gomori lead method. Untreated animals recorded the distribution of acid phosphatase in the primary and secondary spermatocytes as brown granules (Fig. 17). The animals treated with alpha-lipoic acid (served as+control group) showed nearly normal level of the brown granules of acid phosphatase enzyme (Fig. 18).

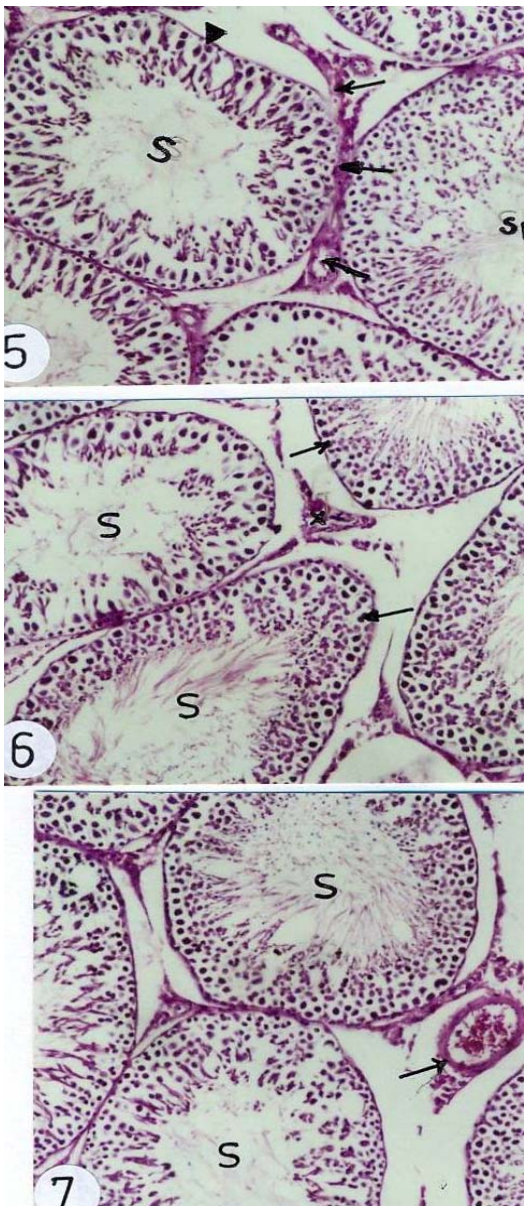

Fig. (5): Photomicrograph of testes sections of rats also treated with pesticide mixture obtained more proliferation within the interstitial cells (arrows).

Fig. (6,7): Photomicrograph of testes sections of rats intoxicated with the pesticide mixture and treated with ALA revealed some amelioration of the hypospermatogonia in the seminiferous tubules (S6) and giant cells between the Leydig cells (arrows 7). Fig. (8): Photomicrograph of testis section of untreated rat stained with masson trichrome stain showed the tunica albuginea normally arranged (arrows).

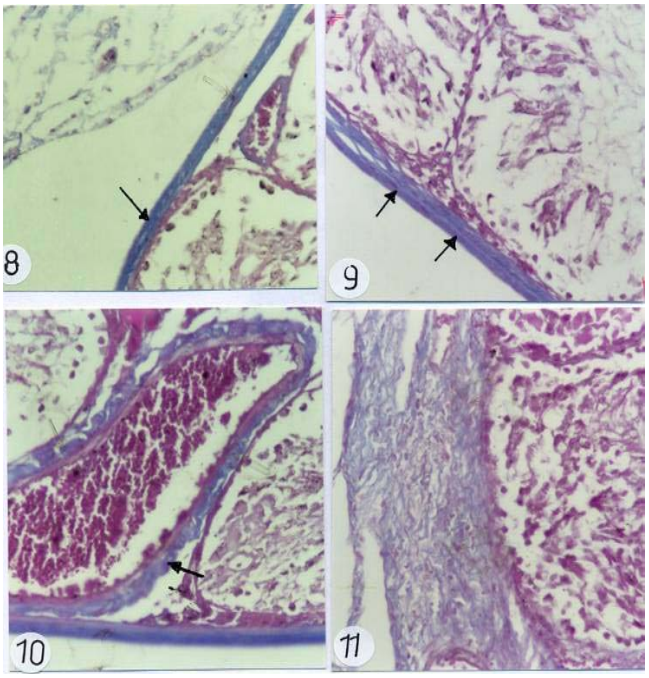

Fig. (9): Photomicrograph of testes section of rat treated with (60mg/kg) alpha lipoic acid showed nearly normal architecture of seminiferous tubules and interstitial cells (arrows).

Fig. (10, 11): Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifose and 1/200 LD50 of fenitithione) showed high degree of fibers around the seminiferous tubules (arrows 10) and around the testis (arrows 11).

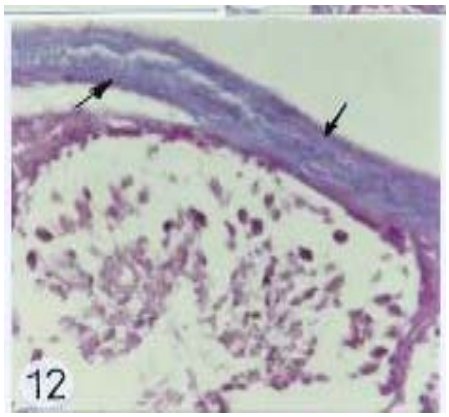

Fig. (12): Photomicrograph of testes sections of rats intoxicated with the pesticide mixture and treated with ALA revealed nearly normal appearance of the fibers with minimal increase comparing to untreated control animals.

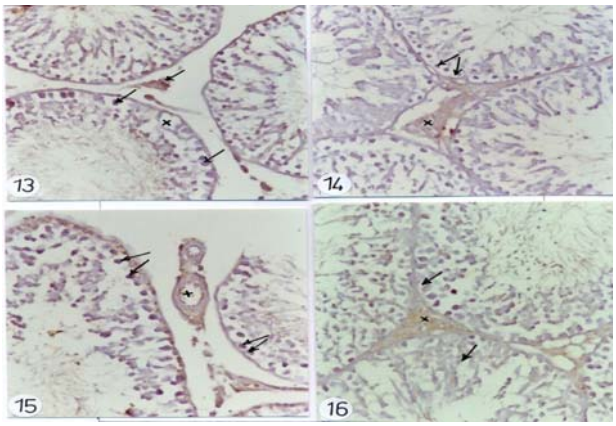

Fig. (13): Photo graph of testis section tissue of untreated rat showed normal positive cells between the spermatogenesis cells (arrows).

Fig. (14): Photomicrograph of testes section of rat treated with (60mg/kg) alpha lipoic acid showed some positive cells within germ cells (arrows).

Fig (15): Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifose and 1/200 LD50 of fenitithione) showed significant increase in the brown positive cells within the primary stages of germ cells (arrows).

Fig. (16): Photomicrograph of testes sections of rats intoxicated with the pesticide mixture and treated with ALA revealed nearly decrease of the positive cells (arrows).

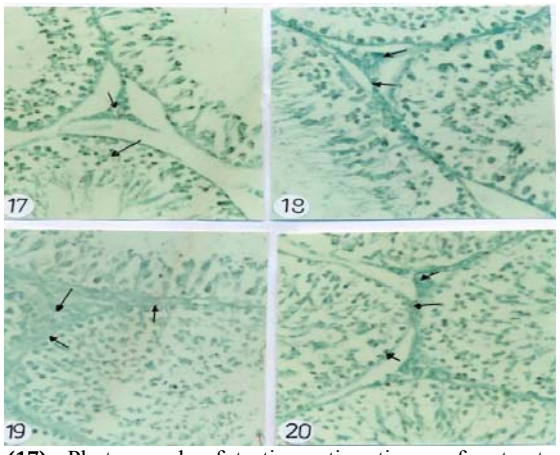

Fig. (17): Photo graph of testis section tissue of untreated rat showed normal distribution of the brown granules of the acid phosphatase enzyme within primary stages of spermatogonia (arrows).

Fig. (18): Photomicrograph of testes section of rat treated with (60mg/kg) alpha lipoic acid showed normal content of the brown granules within germ cells (arrows).

Fig. (19): Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifose and 1/200 LD50 of fenitithione) showed significant depletion in the brown granules within the primary stages of germ cells (arrows).

Fig. (20): Photomicrograph of testes sections of rats intoxicated with the pesticide mixture and treated with ALA revealed nearly normal view of the acid phosphatase enzyme (arrows).

4. Discussion:

The testicular toxicity of insecticides was proven and alternative harmless control strategies should be applied. Insecticides were proven to be induced sever testicular toxicity as shown in the histopathological results which coupled with marked changes of biochemical results. Our results obtained spermatogonial depletion and atrophy due to pesticides toxicities in the seminiferous tubules. We detected desquamated cells in the lumen of seminiferous tubules and vacuolization within germ cells and some tubules contain apoptotic bodies, at the end of treatment, Leydig cells are strongly regressed and spermatozoa are less present in the luminal aspect of the seminiferous tubules. Also thickened basement membrane accompgnied by disappearance of interstitial cells and Leydig cells were proven.

There are several possible mechanisms for the antigonadal actions of organphosphorous in which they may exert a direct inhibitory action on the testis; they may affect the pituitary causing changes in gonadotropin concentration, and may change the concentration of the neurotransmitter acetylcholine (Sarkar *et al.,* 2000; Serin, 2007). The hazardous effect of these pesticides on semen quality continued during the post treatment period, and was dose-dependent (Cakir and Sarikaya, 2005). Therefore Roy *et al.* (2004) showed that chlorpyrifos may exert a suppressive effect on the functional activity of accessory sex glands by decreasing testicular testosterone production following inhibition of pituitary gonadotrophins release (Parashanti *et al.,* 2006). Exposure to low level organophosphorous is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in humans and experimental animals (Sutatos, 1994).

As we shown that, fibrosis correlates with these histological finding, and implicated with the pathological changes. Fibrosis is probably the end result of an inflammatory process. Cell death also occurs spontaneously at various phases of germ cell development and morphological studies have implicated apoptosis in spermatogonial death appears to play a major role during spermatogenesis (Bartke, 1995). The spermatogenesis in mammals depends on testosterone production by Leydig cells in response to stimulation by FSH and LH. FSH increases Sertoli cell synthesis of an androgen binding protein needed to maintain high concentrations of testosterone. LH stimulates testosterone production by the interstitial cells of the testis (Kackar *et al.* 1997).

Sugar (1997) was obtained that acid phospatase enzyme plays an important role in the process of cell metabolism, autolysis, differentiation and many related processes and the dilatation of blood capillaries in between seminiferous tubules obtained upon the result of acid phosphatase enzyme activity. The increase in acid phospahatase enzyme activity could be explained on the bases of enhancement of cell membrane permeability with disturbance in the transphosphorylation process as a result of cellular degeneration (Linder *et al.*1997 and Ibrahim *et al.,* 2003).

The depletion of the enzymatic antioxidative system strengthens the oxidative damage of membranes plays a significant role in cellular damage into the testes (Parasmthi *et al.,* 2005 and Qu *et al.,* 2008). Pesticides may induce oxidative stress, leading to generation of free radicals and alteration in antioxidants, oxygen free radicals, the scavenging enzyme system, and lipid peroxidation (Banerjee *et al.* 1999, Etemadi *et al.* 2002).

Lipoic acid was first postulated to be an effective antioxidant (Biewenga *et al.,* 1997 and Packar *et al.,* 1997). It is able to scavenge reactive species in vitro, though there is little or no evidence that this actually occurs *in vivo.* Alpha Lipoic Acid works both inside the cell and at the membrane level, thereby giving dual protection. Cronan *et al. (*2005) obtained the ability of ALA to create a robust shield on the cell membrane of sperm, along with the liquid that surrounds the sperm indirectly enhance the ability of the sperm to tolerate higher volumes of free radical attack. ALA has also been reported to assist the mitochondria's citric cycle; this in turn will increase the level of reduced glutathione, ATP, TCA cycle enzyme and electron transport chain complex activities (Reddy, 2001 and Henriksen, 2006). ALA regulation of metabolism, increased availability of mitochondrial co-enzymes and improvement of protection of free radicals are thought to eventually lead to a reduced incidence of mitochondria dysfunction, thus ensuring sufficient ATP for sperm movement. (Ibrahim *et al.,* 2008). In conclusion, the toxicity of the pesticides mixture was shown atrophy, fibrosis and increase the incidence of the apoptosis on the testes tissues then, on the fertility and spermatogenesis process. Also, alpha lipoic acid treatment revealed mild ameliorative effect on the pathology of testes.

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Field study on the use of Artemisia cina (Sheih Baladi) and Humates (Humapol-Fis) in the control of Saprolegniosis in fingerlings of Nile tilapias and Mugal cephalus in freshwater fish farms

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Abstract: Saprolegniosis is a fungal disease and it is one of the most causes of economic loss in fish farming industry, affecting all developmental stages. This study was carried out on 300 cultured fingerlings of Nile tilapias and Mugal cephalus from earthen ponds in freshwater fish farms suffered from Saprolegniosis . Diseased fish were subjected to full clinical and postmortem examination. Artemisia cina (Sheih Baladi) and Humates (humic and fulvic acid) were tested for the control of Saprolegniosis affecting fingerlings of Nile tilapias and Mugal cephalus. Artemisia cina L. (A.cina) was used in the form of 5% and 25% stock solutions prepared by pouring boiling water on the herb in a piece of gauze and soaked for 2 hours. The doses were 0.25, 0.5 and 1 ml/l 3 times every an hour for 3 days in fingerlings of Nile tilapias and twice for 2days in fingerlings of Mugal cephalus in earthen ponds. Humates was used as HUMAPOL-FIS dry stock solution in the rates of 5, 10 and 15 g/1000 liter in earthen ponds. Three replicates were used per each treatment and 3 earthen ponds served as control where malachite green or formalin were applied for comparison. Results revealed that A. cina and humates gave the best estimates of viability percentages among the Nile tilapia and Mugal cephalus fingerlings and were safe for fingerlings in the rates of 25% for A. cina and 5 and 15 gm/1000 liter for humates. [Life Science Journal 2010;7(3):125-128]. (ISSN: 1097-8135).

Keywords: Saprolegniosis, Nile tilapias, Mugal cephalus, Artemisia cina and humates

1. Introduction

Saprolegniosis is a continuing problem for aquatic animal culturist causing severe losses of fingerlings fish in earthen ponds and considered as single largest cause of economic losses (Shaheen,1986, Meyer,1991 and El-Ashram, 1997) where it is generally restricted to chronic, steady losses and affected on fingerlings stages (Aly and El Ashram,2000). The control of fungi of the genus saprolegnia has long been a major objective of aquaculturists. Once a fungal infection starts, it can spread rapidly from infected to healthy fish (Pipper et al., 1982 and El-Ashram, 1997). Antifungal are essential for the maintenance of healthy stocks of fish. However, chemical treatment is costly and can itself cause mortality (Jimenez, 1986 and El-Ashram, 1997).For these reasons many researchers have been investigating the use of safer compounds that have no harmful effect on fish and their ecosystem (Hatai, and Hoshiai,1992 and Hussein et al., 2001). Malachite green treated 0.5 mg/l and Formalin (0.1 ml/l) were superior in vitro tests in controlling Saprolegnia in tilapia and mullet fingerlings fish ponds (Fitzpatrick et al.,1995, Laszlo et al., 2002 and Khodabandeh and Abtahi (2006) and Abou El Atta,2008). Malachite green and Formalin are the most potent fungicides that have been prohibited due to their toxicity and persistence in the environment (Meyer and Jorgenson, 1983 and Schreck et al., 1992). Some medicinal plants have a powerful biological effect against fungi, bacteria and even some harmful insects (Diab, A.S. (2002). On the other hand, Humates is considered a potential natural compound used for external fish diseases, fungicide and parasiticide on fish (Hussien, et al., 2010 and Noor El Deen et al., 2010). This study was established to detect the effect of Artemisia cina and humates in preventing Saprolegniosis in fingerlings of Nile tilapia and Mugal cephalus as well as investigating the toxicity or side effects induced by the experimental substances on the treated fingerlings of Nile tilapia and Mugal cephalus.

2. Materials and Methods

2-1-Materials

2-1-1-Fish:

A total number of 150 fingerlings of Nile tilapia and 150 fingerlings of Mugal cephalus were needed. Fingerlings obtained from several earthen ponds were cultured in Lower Egypt fish farms, during the period of October 2008 and November 2009, with different average weight and length. The diseased fingerlings were transported in ice box to Lab. of hydrobiology department, Vet. Div. National Research Centre, Egypt.

2-1-2-The experimental substances:

Artemisia cina and Humapol-Fis; (humic and fulvic acid) were tested against malachite green, formalin and sodium hypochlorite. They were all applied for three successive days on fingerlings of Nile tilapia and Mugal cephalus.

2-1-3-Media:

Sabouraud dextrose agar (SDA) was used for isolation of fungus and was prepared according to (Shahin, 1986).

2-1-4-Stain:

Lactophenol cotton blue stain was prepared

according to (Shaheen,1986).

2-1-5-Experimental design:

LC50 and safety of both wormseed plants and humates solutions were detected by adding 20 ml of Sheih in the rates of 25% as well as of humates solutions in the rates of 5, 10, 15 and 20 g/100 liter in 6 earthen ponds. One pond contained 24 meter of fresh water and served as a control. Fifty fingerlings were added to each of the earthen ponds.Artemisia cina solution (Sheih Baladi) was prepared in the rates of 25% by adding 1 liter of boiling distilled water to beakers containing 50 and 250 g respectively of the herb, wrapped in a piece of gauze. The mouth of the beaker was covered and the herb was left for an hour for proper soaking. The used rates in vitro were 0.25, 0.5 and 1 ml of sheih, 25% per liter of water of fingerlings fish ponds. Artemisia cina solution was added (0.25, 0.5 and 1 ml of sheih, 25% per liter water in the ponds were tested . Humapol-Fis was added (5, 10,15 and 20 gram/ 100 liter of water in the ponds were tested and compared with 1 treatment of formalin (0.1 ml/l of freshwater fish ponds).

2-2-Methods:

2-2-1-Clinical examination:

Three hundred naturally infested Nile tilapia and the same Mugal cephalus were examined for abnormal behaviors and external lesions on the skin, fin according to the method described by Easa and Amin (1987).

2-2-2- Postmortem examination:

Postmortem examination was done on living and freshly dead fish and examination of internal organs.

2-2-3-Mycological examination:

Isolation of fungi was carried out from naturally infected fish, samples were taken from fish showing skin and fin lesions and isolate were collected and inoculated into SDA medium plates and incubated at 20+_2 C for 3-4 days, subculture on the same media was done for purification. Identification of the positive culture was discussed according to Bruno and wood (1994).

3. Results

3-1-Clinical examination:

The main characteristic lesions of Saprolegniosis were in the form of appearance of cotton wool like tufts on the dorsal, tail (caudal, pectoral fins) Plate 1.

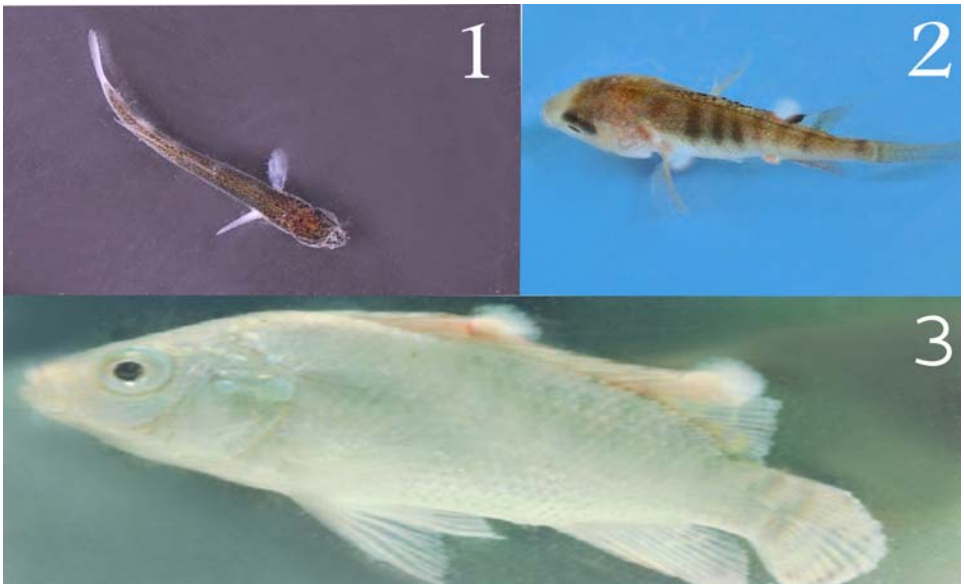

Plate 1: **Showing cotton wool like tufts on the dorsal, tail of fingerlings of Mugal cephalus (1), small fingerling of Nile tilapia (2) and large fingerlings of Nile tilapia (3).**

3-2-Postmortem examination:

The main postmortem lesions were in the form of cotton wool tufts on skin and caudal fin with erosion in tail and pale to grayish gills as well as serious fluid in the body cavity. In addition, intestine was free from any food particles, with dark enlarged liver and distend gall bladder with bile, spleenomegaly and congested kidney.

3-3-Mycological examination:

As shown in table (1); the mycological examination showed an isolation of 164 isolates from 450 samples obtained from 150 infected fish (100 Nile tilapia fingerlings and 50 fingerlings of Mugal cephalus) and different trail of treatment as well as morality rate as showed in table (2).

Table 1: Artemisia cina and humates compared with Malachite green and formalin on Nile tilapia fingerlings and fingerlings Mugal cephalus.

fish	number	organs	Artemisia cina		humates		Malachite green		formalin	
No. Nile tilapia fingerlings	100	No of samples	20	%	20	%	20	%	20	%
		No of isolate	2	1.6	2	1.6	3	2	4	3.2
fingerlings Mugal	50	No of samples	10	%	10	%	10	%	10	%
		No of isolate	1	1.6	1	1.6	6	4	6	4
		Total No of samples	30	20	30	20	30	20	30	20
		Total No of	3	1.6	3	1.6	9	3	10	3.6

The positive colonies on SDA at 20 ºC for 3-4 days started with cysts of long hairs with cottony color after that become grayish then black after 96 hs. incubation.

Table (2): Showing mortality rate of fingerlings after different trail of treatments.

No Of fish	Artemisia cina (0.5 mg/l)		Humates (0.5 g/l)		Malachite green (0.5 mg/l)		Formalin (0.1 ml/l)	
	Mortality after treatment		Mortality after treatment		Mortality after treatment		Mortality after treatment	
	No.	%	No.	%	No.	%	No.	%
100 of Nile tilapia Fingerlings for each	5	5	4	4	25	25	34	34
50 of Fingerlings Mugal cephalus for each	2	4	1	2	20	40	28	56
Total	7	4.5	5	3	45	32.5	62	45

3- 4-Trials of control:

3-4-1- LC50 and safety of Artemisia cina solution and humates were estimated on diseased fingerlings of Nile tilapia and Mugal cephalus sp. Artemisia cina solution in the rate of 25% was found to be safe on fingerlings of Nile tilapia and Mugal cephalus sp, which tolerated and lived for 4 days in the stock solution of 25% and for 6 days in 25% Sheih solution.

3-4-2-Examination the rate of Artemisia cina and humates on fingerlings of Nile tilapia and Mugal cephalus sp was done. Results of Table (1) revealed that Artemisia cina solution and humates in the rate of 0.5 ml/l and applied on fingerlings of Nile tilapia and Mugal cephalus sp than malachite green, and formalin (1gm/10 liter) gave significantly higher figures of survivability% than other doses of Sheih . In addition to results of Table (2) revealed that Artemisia cina solution and humates decrease mortality rate than malachite green, and formalin.

4. Discussions

In Egypt, saprolegnia is considered one of the most important disease causing troubles in freshwater cultured fishes resulting in several economical losses (Easa and Amin, 1987 and Noga, 1996). The clinical signs appear on fish suffered from Saprolegniosis were in the form of grayish white cotton like tufts on dorsal fins, emaciation and death occurred due to secondary infection, toxicity and eye blindness, the fish unable to feed. These results were agreement with (Neish and Hughes, 1980 and El Ashram et al., 2007). The main postmortem lesions were enlargement of liver, spleen and gall bladder which may be due to systemic bacterial infection, these results agree with Hatai,1980 ,Aly et al.,1996 and Abou El Atta,2008.The results of fungal examination showed that isolation of saprolegnia which was isolated with high percentage from gallbladder, kidney and spleen, these results agree with (Marzouk et al., 2003 and Abou El Atta, 2008). Thus, no LC50 was detected for Artemisia cina solution .Humates in a concentration of 15 g/ 100 liter killed all examined parasites after 1h with no deaths of diseased fishes when used in the rate of 5 g/ 100 liter for 4days this agree with El-Ashram,(1997). On the other hand, humates (0.5 g/l) and malachite green (0.5 mg/l) applied on fingerlings of Nile tilapia and Mugal cephalus sp on fingerlings of Nile tilapia and Mugal cephalus, showed relatively higher mortility than Artemisia cina and humates and survivability%. While in case of fingerlings of Nile tilapia and Mugal cephalus sp humates (0.5 g/l) and formalin (0.1 ml/l) revealed significantly higher estimates

of survivability% than other doses of humates (0.25 and 1 g/l) .These results were is agreement with those reported by Gupte et al., (2002), Giesekes et al., (2005), El-Ashram et al., (2007) (Table 2). It could be concluded that, Artemisia cina in the rate of 0.5 ml/l of 25% solution gave the best estimates of survivability% among the examined Mugal cephalus and Nile tilapia fingerlings and recommended for practical application in earthen ponds to replace the currently used chemicals; malachite green and formalin with their well known environmental and public health hazards. Although humates used in the dose of 5 g/100 liter resulted in relatively good results regarding the same measured parameters.

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Diarrhoea in Neonatal baraki kids-goats

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Abstract: A survey was carried out in 130 kids-goats aged from 2 days to 3 month from different private farms in El Mounofia and Kalubia Governorates. Out of these animals, 100 were suffering from diarrhoea. Bacteriological examination of the faecal samples revealed the presence of *E. coli* (58%), *Salmonella*, (27%), and *Shigella* (15%), as the main causative agents of diarrhoea. They were sensitive to common antibiotics and less sensitive to 10% garlic extract and 40% *Hibiscaus subdarifa.* Haematological studies revealed significant decrease in hemoglobin content (Hb), erthrocytic (RBCs) count. On contrary, haematocrit value (PCV %) showed significant increase in affected animals. A significant decrease was detected in the values of serum total proteins, albumin, iron, copper, and growth hormone. On the other hand, there was a significant increase in cortisol hormone, lactate dehydrogenase (LDH), and alkaline phosphatase enzymes. We emphasize that the demonstrated diarrhoea caused many harmful clincopathological effects, reduced growth hormone, and caused severe anaemia in kids-goat. [Life Science Journal 2010;7(3):129-132]. (ISSN: 1097-8135).

Keywords: Kids-goat - kids - diarrhoea - haemogram - Salmonella - *E. coli* -serum biochemistry - LDH - alkaline phosphatase - hormones - trace elements - garlic extract - *Hibiscous subdarfa*.

1. Introduction

With the increasing application of intensive husbandry methods the various causes of ill-thrift in sheep and goats have attracted increasing attention. The results of many investigations have shown that the greatest loss among these species occurs in the neonatal period (Snodgrass and Angus, 1983). Neonatal diarrhoea in kid-goat is a common problem with not very well understood cause (Snodgrass, et al., 1977). This syndrome has been ascribed to a variety of causes such as nutritional imbalance, faulty management and infectious agents (Durham et al., 1979). Infectious diarrhoea affecting kids-goat occurs mainly where intensive systems of breeding which use paddocks, pens and indoor kids-goat sheds are employed. Such systems unless very carefully managed, encourage the progressive build-up of infection (Allan and John, 1987; Aly, et al., 1996 and Angus, et al., 1982).

Aim of the present work to study the cause of diarrhoea, the clinicopathological changes in blood of infected animals and the suitable antibiotic for treatment.

2. Material and Methods

Animals:

One hundred and thirty kids-goat (100 diarrhoeic + 30 apparently healthy as a control group), aged from 2days to 3 months were used in this study. These kids-goats belonged to different localities in El-Mounifia and Kalubeia Governorates and under semi-intensive management system.

Sampling:

All animals were sampled once before administration of any treatment.

Bacteriological Studies:

Fecal samples:

Two faecal samples were taken directly from the rectum of all animal in the investigation. One sample was taken in a clean dry plastic packs for parasitological examination to detect gastrointestinal parasites (Coles, 1986) and the second using sterile swabs for further bacteriological analysis. These swabs were immediately inoculated on Carry and Blair's transport medium and were cultured on selective and differential culture media at 37^{o}C for 24 hours and the isolated colonies were then identified according to Carter (1984) sand Baily and Scott (1990) as follows: Isolated colonies from MacConky's agar plate were examined to be either Lactose fermenting or non-lactose fermenting. Lactose fermenting colonies appeared to be rose pink in color and non-lactose fermenting as pale yellow colonies. Isolated colonies were then examined by Gram staining. Colonies, which appeared as Gram negative bacilli were then described for further identification of Gram negative isolates. These were then subjected to biochemical reactions such as indol production, methyl red Gobes Proskauer test (MR/VP), citrate utilization, hydrogen suiphide production, reaction of triple sugar iron agar (TSI), urease production and oxidase test.

Detection of K99 antigen was performed by slide agglutination test (SAT) according to Baily and Scott (1990), with specific antisera Cryptoporidia were examined in faecal smears on glass slides which were air dried, fixed in methanol and stained with Geimsa stain according to Abou-Zaid and Nasr (1995).

Haematological Studies:

Whole blood samples with EDTA were obtained form the jugular vein for determination of hemoglobin content, haematocrit (PCV%) value, erythrocytic (RBCs) count and total leukocytic (WBCs) count according to Coles (1986).

Biochemical and Hormonal Studies:

Serum samples were used for determination of copper and iron by atomic absorption according to Issac and Kerber (1971). Total proteins, albumin, alkaline phosphatase (ALP), lactate dehydrogenase (LDH) were determined b spectophotomoeter in the rang UV "240 nm". Cortisol hormone was measured according to Kuehn and Burvenich (1986). Growth hormone was measured by special kits according to the method described by Ronge and Blum (1988).

Sensitivity Test:

1. Sensitivity test using common antibiotics:

The following chemotherapeutic agents were used in testing the isolated micro-organisms:

Gentamycin (10mcg/disc), chloramphenicol (30mcg/disc), rifamycine (30mcg/disc), tetracycline (30mcg/disc), ampicillin (10mcg/disc), streptomycin (10mg/disc), nalidixic acid (30 mg/disc), and colistin (10 meg/disc).

2. Sensitivity test using Garlic aqueous solutions:

The isolates were incubated in about 1 0% garlic aqueous solution at 28°C till the colonial broth become evident. The degree of inhibition was compared to control.

3. Sensitivity test using dry *Hibiscus subdarifa* flowers:

The flowers were extracted with 75% ethyl alcohol using apparatus Soxhlet till complete exhaustion occurs. Alcohol was then evaporated to obtain a semisolid extract. Dilutions to 40% were obtained by dissolving the extract in distilled water. The resultant dilutions were used to test microorganisms were streaked with 0.4 mm loop on the extract into the gutter avoiding it over flow on the surface.

Statistical analysis:

All data were subjected to statistical analysis using T- test according to Gad and Well (1967).

3. Results

Results:

Kids-goat were divided after, careful clinical examination and bacteriological examination of the faecal samples into three groups as shown in Table (1).

Clinical Signs:

Diseased kids-goats showed severe depression unable to stand or move and some of them showed sternal or lateral body recumbent. Soft to watery of faeces tinged with mucus or occult blood or both and having putrefied odour. Varying degree of dehydration and severe losses of skin elasticity. Contaminated skin of anal region, rough hairs, dry muzzle, increase of body temperature, pulse and respiratory rates.

Bacteriological Studies:

Bacteriological examination of the faecal samples of diarrhoeic kids revealed that 100 samples were positive for pathogenic bacteria. The distribution of thee indicated that enteropathogic E. Coil and Salmonella constituted the high incidence while Shigella recorded the very lowerest incidence. The increase in packed cell volume (PCV %) reflected the severity of dehydration occurred in diarrhoeic kids with bacterial enteritis in group 2 (infected with E. coli) and group 3 (infected with Salmonella) than in group 4 (infected with Shigella) and apparently healthy kids (group1). This reflects the severity of diarrhoea caused by enterotoxins produced by enterotoxigenic bacteria proliferation in the intestine which lead to toxaemia and that in turn aggravates the dehydrations. The most characteristic features in diarhoeic kids faeces was watery and contained mucus or occult blood or both and was having putrefied odour could explain the high incidence of isolated enteropathogenic E. coli and Salmonella. However the presence o other pathogenic bacteria was also suggested but their incidence was very low as Shigella

Concerning sensitivity test; the result indicate that E. coli and Salmonnela were highly sensitive to gentamycine, chioramphenicol, rifamycine, and tetracycline, less sensitive to ampicillin and nalidixic acid and resistant to streptomycin and colistine. Moreover, E. coli was moderately sensitive to *Hibiscous subdarifa* and garlic solution (Table 2)

Results of haematology and biochemistry:

A significant decrease in haemoglobin content (Hb), erythrocytic (RBCs) count while, haematocrit values (PCV%) and the leukocytic (WBCs) count showed significant increase in affected animals with E. coli (group2) and Salmonelh (group3) than the control healthy animals (group 1) as shown in Table (3).

As shown in Table (4, 5), there were a significant decrease in total proteins, albumin growth hormone, iron, and copper. On the other hand, there was a high level of cortisol hrrnone, lactate dehydrogenase, an alkaline phosphatase in diarrhoeic kids-goat in comparison with the control one.

Table (1): Bacterial examination of faecal samples of diarrhoeic kids-goats

The organism	Number of isolates	% of isolates
E. Coli	47	68.84
Salmonella spp.	28	44.68
Shigellaspp.	12	1.05

Table (2): Results of sensitivity test against different chemotherapeutic agents

Chemotherapeutic agents	Disc concentration	E. coli	Salmonella
Gentamycin	10 mcg	++++	++++
Chlorampheni-col	30 mcg	++++	++++
Rifamycine	30 mcg	+++	++++

Tetracycline	30 mcg	+++	+++
Ampicillin	10 mcg	++	+
Streptomycin	l0mcg	+	+
Naildixic acid	30 mcg	++	+
Garlic aqueous solution 10%	l0%	++	++
Hibiscous extract 40%	40%	++	+
Colistine	10%	+	+

+++ 0.58mm ++ = 0.38mm + = 0.23mm

Table (3): Means ± SE of haemoglobin (Hb) haematocrit (PCV%) and erythrocytic (RBCs) count in both healthy and diarrhoeic kids-goat.

Animals groups	Number of animals	PCV%	RBCs (x106/p.i)	Rb (g/dl)
Group I (control)	30	22.15 ± 0.12	10.20± 0.21	9.80± 0.21
Group 2 (E. coli)	58	41.00 ± 0.01 **	8.24± 0.22**	8.00± 0.15**
Group3 (Salmonella)	27	32.00±0.12**	8.10±0.12**	8.23± 0.84**
Group 4 (Shigella)	15	21.24 ± 0.73**	9.42± 0.30	9.13± 0.12

** = Highly significant at P≤ 0.01 SE = Standard error.

Table (4) Means ± SE of iron, copper, cortisol and growth hormones in both healthy and diarrhoeic kids-goat.

Animals groups	Number of animals	Iron (mg/dl)	Copper (mg/dl)	Cortisol (ng/dl)	Grbwth Hormone (ng/dl)
Group 1 (control)	30	240 ± 2.40	185 ± 3.4	0.098 ± 0.73	11.0 ± 0.08
Group 2 (E. coli)	58	172 ± .53**	130 ± 47**	0.135 ± 0.26**	8.0 ± 0.14 **
Group3 (Salmonella)	27	181 ± 3.50**	134 ± 4.0	0.140 ± 0.30**	7.8 ± 0.25**
Group 4 (Shigella)	15	165± 3.01**	142 ± 2.1 **	0.151 ± 0.42**	7.6 ± 0.35**

** = Highly significant at P≤ 0.01 SE = Standard error.

Table (5): Means ± SE of total proteins, albumin, lactate Dehydrogenase (LDH). alkaline phosphates (ALP) changes in both healthy and diarrhoeic kids-goat.

Animals groups	Number of animals	Total proteins (g/dl)	Albumin (g/dl)	LDH (U/l)	ALP (U/l)
Group 1 (control)	30	9.8 ± 0.40	4.97 ± 0.27	252 ± 30	15.3 ± 0.85
Group 2 (E. coli)	58	8.2 ± 0.37**	3.80 ± 0.24**	263 ± 35 **	18.7 ± 0.40*
Group3 (Salmonella)	27	7.0±0.10**	3.40± 0.72**	271± 14**	18.4 ± 50 **
Group 4 (Shigella)	15	6.8 ±0.78**	2.95 ± 0.73**	263 ± 26**	18.9 ± 0. 4**

** = Highly significant at P≤ 0.01 SE = Standard error.

4. Discussions

Infectious diarrhoea is a common condition affecting kids-goat specially those which are bred under intensive system of breeding in this study. Fecal samples screened the presence of the common enteropathogenic organisms E. Coli, Salmonella species and Shigella which causing diarrhoea. E. Coli seems to be the dominant enteropathogen which plays the major role among diarrhoeic kids goat (Tzipori, et. al., 1981; Angus, et. al., 1982; Carter, 1984; Farid, et. al., 1987 and Rodostits, 1992). Isolation of Salmonella species from diarrhoeic kids-goat confirmed the opinion that Salmonellosis is a sporadic cause of enteritis and cause loss in young kids-goat and buffaloe-calves (Bhullar and Tiawana, 1985). E. Coli and Salmonella were sensitive to garlic 10 %. *Hibiscou.s sabdorifa* flowers 40% sensitive to E. Coli but less sensitive to Salmonella.

The significant decrease in serum total proteins, albumin, iron and copper, in diarrhoeic kids-goat may be referred to the cause of diarrhoea. Where, there was significant increase in bacterial enteritis this could be explained by impaired absorption of these trace elements through the damaged intestinal epithelium resulting from enterotoxins produced by these bacteria in the small intestine (Kasari, 1990 and Aly el. al., 1996). Concerning serum protein and albumin, they showed significant decrease in diarrehoeic kids-goat than the cntrol group. Such drastic reduction may be attributed to diarrhoea, which lower the synthetic power of albumin in the liver due to microorganism. This opinion is supported by finding of Aly, et al. (1996). The significant increase in alkaline phosphatase, lactate dehydrogenase was observed in diarrhoeic kids-goat. Similar results were observed by Sadiek (1987)

A highly significant decrease in serum iron was noticed in diarrhoeic kids-goat, this result agreed with those obtained by Aly, et. al. (1996). The decrease of iron was accompanied by decrease of copper and this lead to anaemia. (Radostitis, 1992).

Concerning cortisol hormone, an increase of this hormone can be considered as an expression of stress and helps the organism to counteract this stress, bacmatological, metallic and endocrine changes enhanced protein catabolism and gluconeogenesis during endotoxaemia (Dvorak, et al., 1974). Growth hormone concentrations tended to decrease in diarrhoeic kids-goat. An effect which was probably in part mediated by tumour necrotic factor (Walton and Cronin, 1989).

We can conclude that a substantial, bacteriological haematologlcal, biochemical, and hormonal changes occur in diarrhoeic kids-goat when the cause of diarrhoea is enterotoxigenic bacteria. This means that we must interfere quickly with therapeutic plan to put in consideration the decrease in damaged intestinal epithelium and supporting the body immune status during infection along side with the traditional electrolyte therapy

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Estimating Of Some Trace Elements In Mineral Water In The Kingdom Of Saudi Arabia

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Abstract: A novel sensor was developed for simultaneous detection of Pb, Cd, Cu and Zn concentration based on the differential pulse anodic stripping voltammetry techniques. Response (DPSV) performed on a hanging mercury drop electrode (HMDE). The estimation of Pb, Cd, Cu, Zn concentration in the mineral water of Al – Qasim, Hana Al Qasim, Najran, Nova, Safa Makah, and Mozn Jazan drinking water in the Kingdom of Saudi Arabia was accomplished. [Life Science Journal 2010;7(3):133-137]. (ISSN: 1097-8135).

Keywords: mineral water; voltammetry; Saudi; trace elements.

1. Introduction

The importance of the hygienic, safety, and validity of drinking water, was conceded by James. B. Grant the chief executive of The United Nations (UNICEF) who said: "It was well known that it is for a long time the majority of diseases in the third world were attached by one way or another by the shortage of purified water and by the hygienic reasons , so we have to attached to our believes in the importance of providing the purified water and in the importance of hygienic reasons".

The chemical analysis of drinking water is important because we should know about what it contains from mineral elements, pollution's degree, contaminated materials, and the most important mineral impurities.

The studies show that Cadmium (Cd) causes a lot of serious health problems and if it accumulates in the kidneys with a high critical amount it will affect them and weaken their functions to the point that they will probably fail to do their duty. On the other hand Cadmium is responsible about the blood pressure and affects heart making it inflate, also poisons of Cadmium affects from its the metabolism (Demetriades et al., 2004, He et al., 2007, Portugal et al., 2007)

While Lead (Pb) which is considered as an important and famous polluted substance in the aquatic environment, is very harmful for body health. When it transmits to human body it will ruin brain cells and causes slow death .The danger of lead due to the inability of body to get rid of its accumulates in the body causing a lot of physical, and healthy risks.

The important symptoms that appear is poising by lead, abdominal cramps and bouts of diarrhea, constipation and general weakness , paralysis of hands or feet, feeble eyes, sleeplessness, bouts of nervousness, depression, convulsions, fainting, nervous irritation, and embryo deformation (Sonthalia et al., 2004).

Copper (Cu) is an essential trace element. Copper is a vital part of several enzymes (e.g., ferroxidases, cytochrome coxidase, superoxide dismutase, tyrosinase, lysyl oxidase, and dopamine beta hydroxylase). The absorption depends on the amount ingested, its chemical form, and the composition of other dietary components such as zinc. Drinking water may contribute significantly to the daily copper intake because of the widespread use of copper pipes. Absorption is regulated by homeostatic mechanisms in the liver, and biliary excretion increases when copper is in excess. No quantitative data on pulmonary absorption are available (Ellingsen et al., 2007). The increasing of Copper concentration beyond the limits could cause acute intestinal effectiveness especially, nausea, diarrhea, and abdominal pains with unacceptable taste which may lead to aggressive erosion on metal bowls and Pipes (Herzog and Arrigan, 2005).

Zinc (Zn) leads to poison and irritate the digestive system (upset stomach), causing a lack of absorption of copper, and body temperature will be raised which will affect body immunity (Khun and Liu, 2009).

Stripping analysis has been proved a powerful and versatile technique for the determination of trace heavy metals in various samples (Wu et al., 2008).

2. Materials and methods.

Gathering samples:

Bottles water samples were chosen from Al Qasim, Hana Al Qasim, Najran, Nova, Safa Holy Makkah, and Mozn Jazan drinking water for one-year validity (Al-Saleh, 1996).

Preparing of samples:

The studied samples were acidified by adding one millimeter of intensified Nitric acid, HNO_3 (70%).

The apparatus used in the study:

The concentration of trace elements were measured by Polargraph instrumental 746 VA trace analyzer with 747 VA stand or from Metrohm company. The information storage is done by a computer, from Toshiba company 757 VA computracy joined with the device.

3. Results and discussion

Table 1 shows that the concentration of elements which is under study in the mineral water for the chosen six factories. They have been analyzed using SPSS program, at significant ($p<0.01$).

Table 1. The concentration of studied elements in the mineral water in the six factories.

Factory	Elements concentration Mean ± S.D.			
	Pb	Cd	Cu	Zn
Hana - Al Qassim	6.2049 ±0.311	0.056± 0.0036	66.5397 ±0.606	120.1086± 0.6303
Al-Qassim	2.3794 ±1.18	0.0376± 0.0146	40.1828 ±2.159	136.7745± 1.9165
Nova	13.455 4±0.21	0.0811± 0.0031	64.8513 ±2.0241	193.2588± 17.9635
Safa Makkah	4.5461 ±0.94	1.8993± 0.2493	66.3968 ±3.199	199.3590± 0.2135
Najran	18.007 7±2.48	0.0615± 0.0003	33.703± 6.1549	128.161± 4.9022
Mozn Jazan	4.643± 0.9435	0.0564± 0.0038	58.4476 ±2.914	143.1204± 9.879

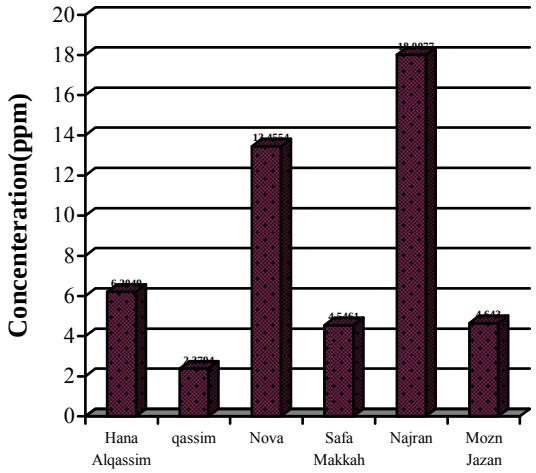

Figure 1.Concenteration of Lead(Pb) in mineral water of six factories

The highest concentration was found with Pb noticed is (18.0077 ppm) in Najran drinking water, while Al-Qasim has the lower concentration it reached (2.3794ppm)(van staden and Taljaard, 2004); the order is: Najran > Nova >Hana Al- Qasim> Mozn Jazan> Safa Holy Makkah >Al-Qasim.

Also the study showed that the highest concentration Cd was in Holy Makkah drinking water (1.8993ppm) then Nova drinking water where (0.0811ppm), then Najran drinking water (0.0615ppm) then Mozn Jazan drinking water (0.0564ppm), then Hana Al Qasim drinking water (0.056ppm),then Al Qasim drinking water (0.0376ppm)(Bakker and Pretsch,2005).

The study approved that the highest concentration of Cu was (66.5397ppm) in Hana Al Qasim drinking water then Safa Holy Makkah drinking water (66.3968ppm), then Nova drinking water reached to (4.8513ppm) after that Mozn Jazan drinking water reached to (58.4476 ppm) , then A l Qasim drinking water reached to (40.1828 ppm). Finally Najran drinking water where concentration reached to (33.703ppm) (Güler and Alpaslan, 2009).

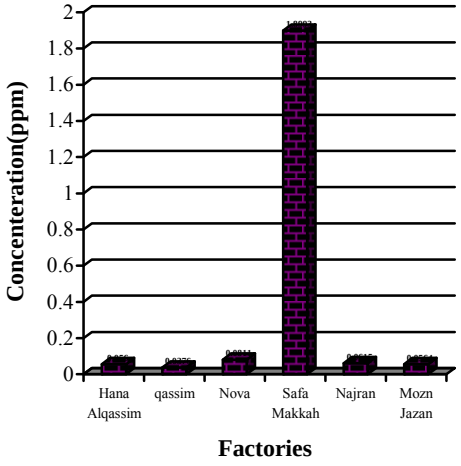

Figure 2.Concenteration of (Cd) in mineral water of six factories.

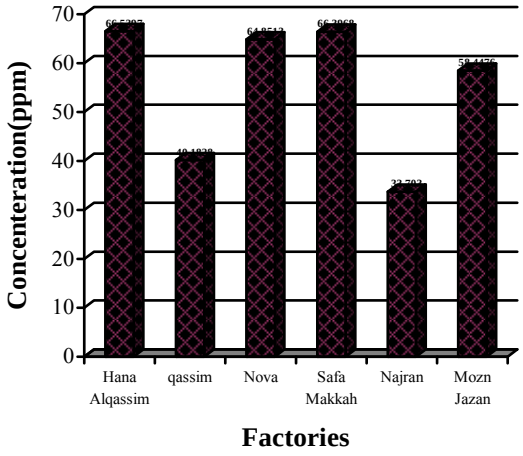

Figure 3.Concenteration of Copper (Cu) in mineral water of six factories.

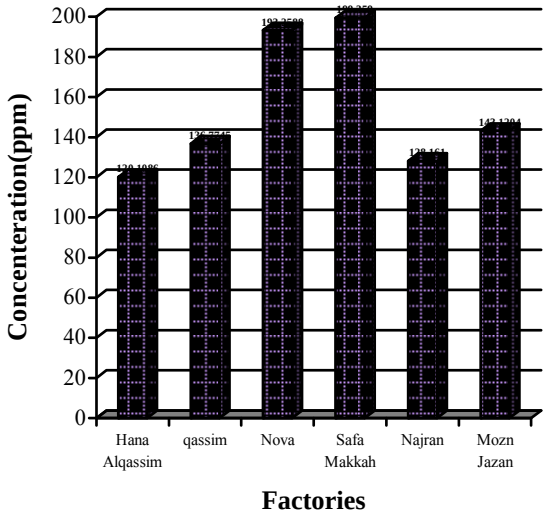

Figure 4.Concenteration of Zinc (Zn) in mineral water of six factories.

Also the study approved that the highest concentration of Zn was found in Safa Holy Makkah drinking water which reached (199.3590 ppm) then Nova drinking water reached to (193.3588 ppm) then Mozn Jazan drinking water reached to (143.1204 ppm), after that Al Qasim drinking water reached to (136.7745 ppm), after that Najran drinking water reached to (128.161 ppm), finally Hana Al Qasim drinking water reached to (120.1086ppm) (Emmanuel et al., 2009).

Also the study clarified the differences between elements concentration, so that it can be seen in :

1. ***Hana Al - Qasim*** water ,the highest concentration Zn element is found ,that it reached to (120.1086ppm) where the less concentration was Cd element , that it reached to (0.0560ppm . Cu reached to (66.5397ppm) , following that ,Pb element where it reached (6.2049ppm).
2. ***Al Qasim*** drinking water concerning Cd, it was the lowest concentration within (0.0376ppm) following that Cu within (40.1828ppm) after that Zn within (136.7745ppm).
3. ***Nova*** drinking water, in that the highest concentration Zn was within (193.2588ppm) ,and it was lower in concentration Cd element within (0.0811ppm).
4. ***Safa Holy Makkah*** drinking water ; it was the lowest concentration Pb element within (4.5461ppm), and the highest concentration Zn within (199.3590ppm).
5. ***Mozn Jazan*** drinking water concerning Cu within (58.4476ppm) and Cd concentration reached to (0.0564ppm) while the lowest concentration was in Pb element where it reached to (4.6430ppm) and the highest concentration was Zn element within (143.1204ppm).
6. ***Najran*** drinking water; was the lowest concentration of Cd element within (0.0615ppm), and the highest concentration of Zn within (128.161ppm).

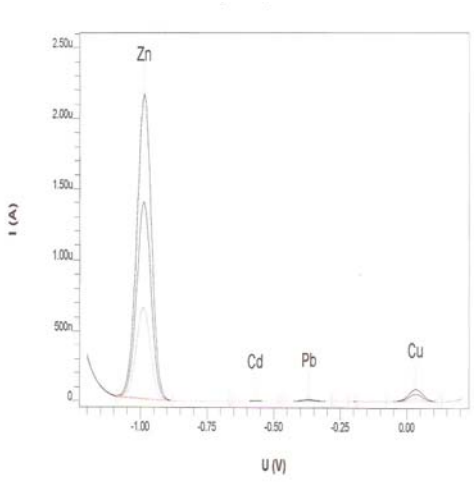

Figure 5. Voltammogram of the trace elements (Cu,Pb,Cd,Zn) in drinking water.

Also it is clarified from the Figure 5. Voltammogram of the trace elements (Cu, Pb, Cd, Zn) in drinking water, that the highest concentration is of Zn element in all mineral water that is under the study , then Cu element concentration then Pb element concentration and finally Cd. The study of the correlation coefficient between elements shows the following:

(1) ***in Hana Al Qasim*** drinking water there is a strong positive correlation between Pb and Cd (r = 0.727), also correlation coefficient is strong positive between Zn and Cu (r = 0.886).

(2) ***in Al Qasim*** drinking water a strong positive correlation between Zn and Cu (r = 0.838) is found ; also the correlation coefficient is strong positive between Pb and Cd (r = 0.956).

(3) ***in Nova*** drinking water , it was found that there is a strong positive correlation between Pb and Cd (r = 0.66), and also the correlation is strong positive between Zn and Cu (r = 0.90).

(4) Also the correlation coefficient is strong positive between Cu and Cd (r = 0.925) and between Zn and Pb (r = 0.679) in the study of ***Safa Holy Makkah*** drinking water .

(5) the correlation coefficient is strong positive (r=0.987) between Pb and Cd ;and strong positive between Zn and Cu (r = 0.885) in the study of ***Mozn Jazan*** drinking water .

(6) ***in Najran*** drinking water the correlation coefficient is weak (r = 0.014) between Cd and Cu under the study , while it was strong between Zn and Pb (r = 0.958).

On the other hand , for the studied elements, it can be stated that :

The study of the concentration of Pb element in different bottled drinking water samples, showed that the correlation coefficient is strong positive between the concentration of Pb element in Al Qasim drinking water and the concentration of Pb element of Najran drinking water (r = 0.965).

Also the correlation coefficient is strong and positive (r = 0.937) between Pb element concentration in Al Qasim drinking water and the Pb element concentration in Safa Holy Makkah drinking water , while correlation is medium positive (r = 0.619) between Pb element condensation in Al Qasim and Hana Al Qasim.

The study also, showed that the correlation is weak (r = 0.059) between Pb element condensation in Al Qasim drinking water and Nova drinking water, while correlation is medium and positive (r = 0.681) between Pb element in Najran drinking water and Hana Al Qasim drinking water.

Also the correlation is weak (r = 0.20) between Pb element in Najran drinking water and Nova drinking water , while correlation coefficient is strong and positive (r = 0.943) between Pb concentration in Najran drinking water and Pb concentration in Safa Holy Makkah drinking water.

The study explained that the correlation coefficient is weak (r = 0.045) between Pb concentration in Nova drinking water and in Safa Holy Makkah drinking water.

The study of the correlation coefficient for Cd element among the samples of the studied factories clarified that the correlation coefficient is strong and positive between Cd element concentration in Al Qasim drinking water and Cd element concentration in Hana Al Qasim drinking water (r = 0.967), in Safa Holy Makkah drinking water (r = 0.978) , and in Nova drinking water (r = 0.990).

While the correlation coefficient is weak between Cd element concentration in Najran drinking water and in Mozn Jazan (r = 0.224). The study also showed that the correlation coefficient is strong and positive between Cd element concentration in Hana Al Qasim drinking water , and Nova drinking water (r = 0.974).

The study approved that the correlation coefficient is positive and strong between Cd concentration in Safa Holy Makkah drinking water, Cd element concentration in Hana Al Qasim drinking water (r = 0.955), and Nova drinking water (r = 0.969).

The study approved that the correlation coefficient is positive and strong among Cu element concentration in Mozn Jazan and Cu element in Hana Al Qasim (r = 0.964), in Safa Holy Makkah (r = 0.967) in Nova drinking water (r = 0.992).

The study clarified that the correlation coefficient between Cu concentration in Najran and Cu concentration in Al Qasim drinking water (r = 0.914) is strong and positive. Also the study clarified that the correlation coefficient is positive and strong between Cu in Nova drinking water and Hana Al Qasim drinking water (r = 0.967) .

Also the correlation coefficient between Cu element condensation in Safa Holy Makkah and Hana Al Qasim was positive and strong (r = 0.983).

The study confirmed that the correlation coefficient is strong between Zn element concentration in all of Nova drinking water , Najran drinking water (r = 0.971) , Mozn Jazan drinking water (r = 0.944) , Zn in Hana Al Qasim (r = 0.994) , and in Safa Holy Makkah drinking water (r = 0.652).

The study also clarified that the correlation is strong and positive between Zn concentration in all of : Najran drinking water , Mozn Jazan drinking water (r = 0.971) , Hana A l Qasim (r = 0.971) , and Safa Holy Makkah drinking water (r = 0.704). Also the study clarified that the correlation coefficient is positive and strong between Zn element concentration in Safa Holy Makkah , Mozn Jazan drinking water (r = 0.759) , and Hana Al Qasim (r = 0.613).

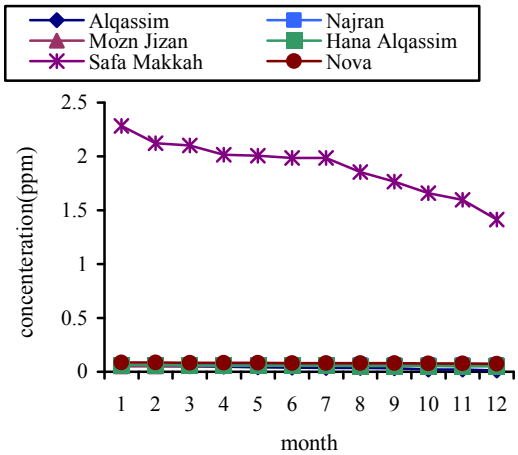

Figure 6.Concenteration of Cd in mineral water of six factories

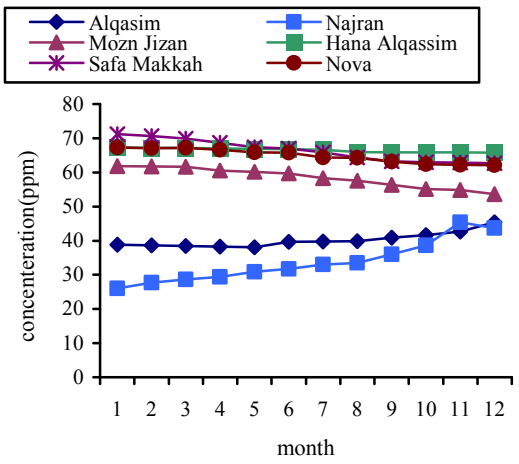

Figure 7.Concenteration of Cu in mineral water of six factories

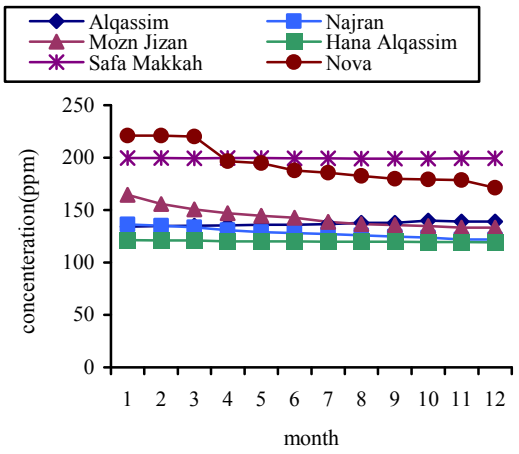

Figure 8.Concenteration of Zn in mineral water of six factories

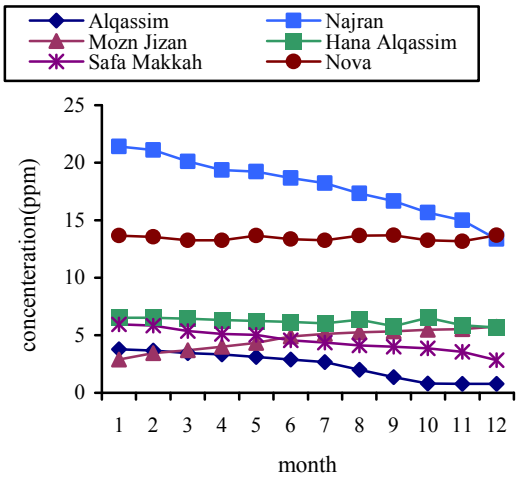

Figure 9.Concenteration of Pb in mineral water of six factories

Figures (6 - 9) show the study of the timing effect on concentration of trace elements which is under study in the mineral water for the chosen six factories. The obtained data from the figures show that the Pb element concentration in Al-Qasim drinking water is reduced from (3.771ppm) to (0.7711ppm) and also it is

reduced from (21.421ppm) to (13.3720ppm) in Najran drinking water (Dragoe et al.,2006). Pb concentration is reduced in Mozn Jazan from (5.762ppm) to (2.899ppm), and Pb concentration is reduced in Hana Al Qasim from (6.535ppm) to (5.662ppm). The study clarified that Pb element is reduced in Safa Holy Makkah from (5.937ppm) to (2.847ppm), Pb element concentration is reduced in Nova from (13.698ppm) to (13.159ppm) and also the situation as it concerning to Cd,Cu,and Zn (Asubiojo et al., 1997).

4. Conclusion

The estimation of Pb ,Cd ,Cu ,Zn concentration in the mineral water of Al – Qasim , Hana Al Qasim , Najran , Nova , Safa Makah , and Mozn Jazan drinking water was accomplished using voltametry techniques .The highest concentration of Pb is in Najran drinking water. Also the study showed that the highest concentration Cadmium is in Safa Holy Makkah drinking water. It approved that the highest concentration of Cu is in Hana Al Qasim drinking water. The study approved also that the highest concentration of Zn is in Safa Holy Makkah drinking water. The obtained results were lower than the average range of these elements in the maximum concentration as they were allowed to be by The World Health Organization (WHO) (Öztürk and Yilmaz, 2000).

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Aspects of Egg Laying in Indian Robin (*Saxicoloides fulicata*)

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Abstract: Several aspects of the breeding biology of wild birds have been studied thoroughly. However, information on patterns of egg laying is lacking for most passerines species. Using direct observations of individuals of Indian Robins (*Saxicoloides fulicata*), we documented the timing and duration of egg laying and behaviour of males and females around the time of laying in a tropical study area of Haridwar (29^0 55' N, 78^0 08' E), India. The mean laying time was SR + 41.4 ± 8.9 min (range: SR + 24 - 58 min) and did not vary with the laying order. The mean duration of laying bouts was 16.9 ± 5.37 min and did not vary significantly for the laying of successive eggs. Females seemed equally active before and after egg laying indicating laying times to be determined primarily by physiological mechanisms involved in egg formation, such as hormone surges and ovulation. In most cases, females were accompanied by their mates when approaching the nest to lay. After leaving their nests following laying, females were immediately joined by their mates or they flew directly to them. Such observations suggest that both sexes may be guarding their pair bond against divorce by either member of the pair.
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Key words: Bout duration, egg laying, Indian Robin, *Saxicoloides fulicata*, sunrise

1. Introduction

Estimates of laying times and duration, combined with observations of pair interactions around the time of laying have been known to contribute to our understanding of several aspects of avian biology, such as energetics, hatching asynchrony, the timing of brood parasitism and copulations, and patterns of mate guarding (McMaster *et al.*, 2004). However, such aspects have been poorly studied for most passerine species and the results vary from species to species (McMaster *et al.*, 1999). For example, based on the handful of studies to date, passerines appear to lay either within a restricted period around sunrise or later in the day with significant variation in laying times (e.g. Weatherhead *et al.*, 1991; Oppenheimer *et al.*, 1996; Gill, 2003). Similarly, the time it takes to lay an egg, or laying bouts, varies considerably from just a few seconds to over an hour (Nolan, 1978; Muma, 1986; Peer and Sealy, 1999). In addition, there is almost no information on the interactions between females and their mates before, during, and after laying (Gill, 2003).

In the present study, we gathered information on the timing of egg laying in Indian Robin (*Saxicoloides fulicata*). The objectives of this study were:
(1) to determine egg laying times and laying-bout durations, and (2) to determine the behavior of males and females around the time of laying.

2. Materials and Methods

2.1 Study period and area

We observed individuals of Indian Robin from April to June 2010 within a 12-km radius in Haridwar (29^0 55' N, 78^0 08' E), Uttarakhand, India. A total of forty laying instances were observed in 12 nests.

2.2 About the species:

The Indian Robin, a territorial bird species, belongs to the Muscicapidae family. It is widespread in South Asia (Ali and Ripley 1998). It is sexually dimorphic in plumage with the male being mainly black while females are brown in most parts of the body (Grimmett *et al.*, 1998). Juvenile birds are much like females but the throat is mottled (Rasmussen and Anderson, 2005).

This species is found in open stony, grassy and scrub forest habitats (often close to human habitation). Pairs are mainly seen in dry habitats and are mostly absent from the thicker forest regions and high rainfall areas. All populations are resident and non-migratory (Ali and Ripley, 1998). The breeding season varies according to region and usually begins with the first rains (Betts, 1951). Peak breeding in the study area was observed during May (personal observation). Males sing during breeding season and display by lowering and spreading their tail feathers and strutting around the female, displaying their sides and fluffing their undertail coverts (Thyagaraju, 1955). Three to four eggs is the usual clutch (Oates, 1890). Only the female incubates; however, provisioning to young is carried by both the sexes (George, 1961; Ali and Ripley, 1998).

2.3 General methodology

We arrived at study sites during dawn, before awakening of males and females and immediately checked the nest contents. When a nest had one or two eggs, we watched the nest following morning to record laying of consecutive eggs of the clutch. On several occasions, we located the nests under construction, and in such nests we were able to observe the laying of first egg as well. Nests were observed from considerable distance without affecting the pair's behavior. We recorded the time that the female entered the nest to lay and whether the male accompanied the female on her return. Once the female entered the nest, we watched it until the female exited, recorded the time, and then followed the female to determine whether the pair reunited. Nests were re-checked after the laying bout to confirm the presence of a new egg. The duration of the laying bout was calculated by subtracting the time females entered the nest to lay from the time they left it after laying. The midpoint of the

laying bout was considered as laying time. All behaviors were recorded to the nearest minute.

We present times of day as minutes relative to sunrise, which are represented as SR - or SR + depending on whether the event occurred before or after sunrise, respectively. Sunrise times for Haridwar were obtained from the website: www.timeanddate.com. Results are reported as means±SD. We used the non-parametric Kruskal-Wallis test to examine variation in laying timing and bout durations among successive eggs within the clutches (Rao and Richards, 2007).

3. Results

The mean laying time for the Indian Robin was SR + 41.4 ± 8.9 min (range: SR + 24 - 58 min) (Table 1) and did not vary with the laying order ($H = 0.955$, $df = 3$, $P>0.05$). The duration of laying bouts ranged from 9 to 33 min with a mean laying bout duration of 16.9 ± 5.37 min (Table 1). The bout duration also did not vary significantly for the laying of successive eggs ($H = 0.947$, $df = 3$, $P>0.05$).

In 17 out of 40 occasions, female Indian Robins were observed foraging on ground nearby the nests before laying. For seven occasions females sang during aggressive interactions with floater or neighbouring females before laying. As soon as a focal female observed any female inside or nearby the territorial boundary, she sang and chased it for a long distance. All such activities of females (such as foraging, calling, chasing intruder females) were observed following egg laying also.

For 37 occasions, females were accompanied by their mates when approaching the nest to lay while in remaining occasions females approached the nest alone. When females were in their nests their mates perched or foraged usually within 3-15 m of the breeding nest. On 32 occasions, these close perching males spontaneously sang loudly when females were in the nest to lay. After leaving their nests following laying, females were immediately joined by their mates (n = 31) or they flew directly to them (n = 7). However, in two instances females flew out of sight once they left the nest, and we were not able to determine when the pair came into contact.

Table 1. Egg laying time and bout duration in Indian Robins (*Saxicoloides fulicata*)

Eggs	Laying time relative to sunrise (SR)	Bout duration (min)	N
I egg	SR+44.42 ± 9.05 (SR +29 to +53)	16.57 ± 4.75 (10-22)	7
II egg	SR+40.33 ± 7.87 (SR +27 to +54)	16.58 ± 5.77 (10-27)	12
III egg	SR+41.16 ± 10.61 (SR +24 to +58)	17.75 ± 2.92 (14-24)	12
IV egg	SR+40.77 ± 8.84 (SR +30 to +55)	16.55 ± 8.06 (9-33)	9
All eggs	SR+41.4 ± 8.94 (SR +24 to +58)	16.92 ± 5.37 (9-33)	40

4. Discussion

The Indian Robins laid within a restricted period of the day, about forty minutes after sunrise. The timing of egg laying varies considerably among species. For example, species may lay eggs before sunrise (Brown-headed Cowbirds *Molothrus ater*, Scott, 1991; McMaster *et al*., 2004), shortly after sunrise (Red-winged Blackbirds *Agelaius phoeniceus*, Muma, 1986), about two hrs after sunrise (Gray Catbirds *Dumetella carolinensis*, Scott, 1993) and throughout the day (Dusky Flycatchers *Empidonax oberholseri*, Oppenheimer et al., 1996).

The laying time of Indian Robin coincided with the intense period of the daily activities. For example, females were equally active in various activities such as singing, foraging, interacting with intruder females etc. before and following laying. It has been suggested that egg laying should be less likely to occur at such a time because of the potential risk of damage to an oviducal egg or as a result of energy constraints (Schifferli, 1979; Oppenheimer *et al.*, 1996). However, these factors appear to have a negligible influence on the time of laying in Indian Robins. Like Indian Robins, inconsistencies between time of laying and female activity have been noted in other species also. For example, in American Robin (*Turdus migratorius*), an early morning to late afternoon layer, no differences in female activity were observed before or after laying (Weatherhead *et al.*, 1991). Similarly in European Starlings (*Sturnus vulgaris*), most laying occurred near the end of the peak period of morning social activities (Feare *et al*., 1982). These considerations suggest that laying times per se may not be the focus of natural selection in some species. Rather, laying times appear to be determined primarily by physiological mechanisms involved in egg formation, such as hormone surges and ovulation (Oppenheimer *et al.*, 1996).

The mean laying bout duration of Indian Robins was 16.92 ± 5.37 minutes which appears much shorter than a number of bird species studied so far (see McMaster *et al.*, 2004). However, the reason for this is unclear. It has been suggested that species that lay later in the day have longer laying bouts, whereas species that lay early may have been under greater time constraints of foraging than those lay later (McMaster *et al.*, 2004). However, we frequently observed female Indian Robins foraging before laying and throughout the day probably because of easy accessibility of their prey.

Most female Indian Robins (>90 % laying occasions) were accompanied by their mates to the nest before laying; males foraged close to the nest during laying, and pairs re-united shortly after laying. Weatherhead *et al.*, (1991) speculated that the time of laying may reflect selection that favors a particular time for fertilization, which in turn may reflect the best time for copulation. Early-morning laying has been considered the optimal time for copulation to take advantage of the female's insemination window (Cheng *et al.*, 1983). Consequently solicitations, copulations, and mate guarding have been expected to be most frequent shortly after laying. Thus, an obvious interpretation of the behavior of the male Indian Robins is this that males are guarding their fertile mates from the copulation attempts of extra-pair males. In some cases (n = 7), where males did not join the female immediately, females sought out their mates after

laying. Such observations suggest an alternative explanation that both sexes may be guarding their pair bond against divorce by either member of the pair (Hall, 2000; Gill, 2003).

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Retrotransposon *Tto1* in tobacco was activated by the implantation of Low-energy N^+ ion beam

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Abstract Tobacco retrotransposon *Tto1*, one of a few active retrotransposons of plants, has been shown to be activated by plant tissue culture (protoplast), wounding stress and methyl jasmonate. Low-energy ion beam, a kind of mutagen, can induce many kinds of botanical mutants. The relation between the retrotransposon *Tto1* and stress of the ion implantation was interested to many researchers focused on idea that whether *Tto1* was activated by implantation of the low-energy ion beam or not. Here，quantitative real-time PCR were used to investigate whether the implantation of ion beam increased the copy number of the *Tto1* in tobacco genomic or not, clustering according to the banding pattern generated by IRAP with primers to retrotransposon *Tto1* was used to investigate the genetic polymorphism between the tobacco population implanted by the ion. The results showed that the copy number of *Tto1* in some treated individuals was creased 10 folds in the second euphylla and more 2 folds in petal. Analysis of the clustering UPGMA method showed that radiation of the ion caused great dissimilarity (dissimilarity coefficient >0.6) between partial treated samples and the controls. The great genetic dissimilarity based on the retrotransposon *Tto1* and the increased copy number of the *Tto1* implied that *Tto1* was also activated by the exposure to the implantation of ion. The transposition of *Tto1* takes place in both the somatic cell and the apical meristem cell, and then, the increased copy number can be transferred from the apical meristem cell of the plantule to the differential organs (here petal is showed). These findings are discussed in the role of the transpositional *Tto1* played in response to the implantation of the low-energy ion beam. [Life Science Journal 2010;7(3):141-147]. (ISSN: 1097-8135).

Keywords: Retrotransposon *Tto1*; *Nicotiana tabacum L.*; Low-energy ion beam; Transposition

Introduce

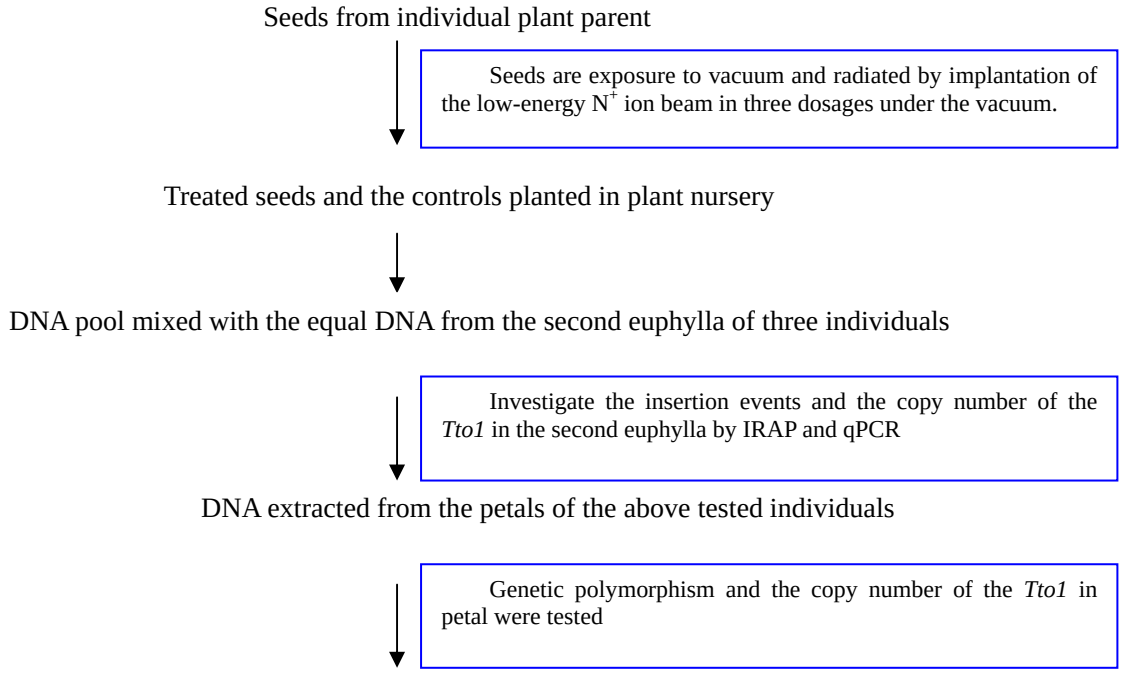
Transposable elements (TEs), being DNA fragments which can be transposed within chromosomes in cells (Feschotte C et al. 2002). Retrotransposons are ubiquitous in plant genomes (Kubis SE et al. 1998; (Kumar A and Bennetzen JL. 1999). Many retrotransposons are integrated in either a transcriptionally or transpositionally incompetent state. However, some quiescent retrotransposons can be activated by wounding, oxidative stress and pathogen infection, and even in plant tissue culture (Feschotte C et al. 2002; Vicient CM et al. 2001; Meyers BC et al. 2001; Grandbastien MA. 1998). Retrotransposon populations are highly heterogeneous, showing great differences in copy number and genomic localization even between closely related species (Carlos MV et al. 1999), Hence several marker systems based upon retrotransposons have been developed. Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), two excellent marker systems based upon the copy numbers and the location of the retrotransposons, can be used to detect any insertion event, which require neither restriction enzyme digestion nor ligation to generate the marker bands. The methods can distinguish between barley varieties and produce "fingerprint patterns for species across the genus (Kalendar R et al. 1999). IRAP and REMAP Markers derived from Tos17, a copia-like endogenous retrotransposon of rice, were used to identify genetic similarity among 51 rice cultivars (Castelo J S B et al. 2007). The IRAP and REMAP techniques can be used separately or combined for a more complete genome survey, and they are excellent sources of polymorphic markers. IRAP/REMAP proved to be as reliable molecular markers as AFLPs, they also bring additional information, showing a great potential use in genome assessments for fingerprinting, mapping and diversity studies (Sonia C. et al. 2007, Andrew J F.1999)

Ttol is 5.5 kb long and has features typical of retrotransposons in tobacco (*Nicotiana tabacum L*), it is the first demonstration of activation of a plant retrotransposon by tissue culture. The copy number of *Ttol* increased 10-fold in established protoplast formation cell lines; it also increased in plants regenerated from tissue cultures and in transgenic plants (Hirohiko H et al. 1993). Functional analysis of the *Tto1* showed that it involved in re-activation by tissue culture, wounding, and treatment with elicitors. *Tto1* has been implicated in the expression of phenylpropanoid synthetic genes in response to defense-related stresses (Kazuhiko S et al.2000).

The mutagenic effect of low-energy ion beam implantation on cereal seeds, such as *Oryza sativa*, *Triticum aestivum* and soybean, has been substantiated by many studies in the past three decades. Many attractive biological effects occurred when the seeds of crops had been radiated by ion beam (Review, Feng HY et al. 2007). Up to now the exact mutagenic mechanism of the low-energy ion beam implantation on cereals has not been fully understood, especially the mechanism of the deleted and inserted DNA of the chromosomes in cell after implantation of the ion. Radialization of the ion beam can affect the transcriptional and transpositional activity of the retrotransposon *Wis2-1A* in wheat (Ya HY et al. 2007). Here we address the idea whether the implantation of low-energy ion beam re-activates transpositional acitivty of *Tto1* in tobacco or not. To test the idea, IRAP and qPCR (Quantitative real-time PCR)

were used to investigate genetic polymorphism (insertion event) and the copy number of the *Tto1* in genome exposure to the implantation of the ion respectively because the copy number and the genetic polymorphism are related to the transposition of the retrotransposons.

Tobacco is the self-pollinated plant, the seeds from one individual plant parent, which had the heredity homogeneity, were implanted by low-energy N^+ ion beam in there dosages. The following is the testing strategy.



Investigate genetic polymorphism (insertion event) and the copy number of the *Tto1* in genome exposure to the implantation of the ion and vacuum, probe genetic polymorphism of *Tto1* that caused by N^+ ion beam and vacuum, test the idea whether the implantation of low-energy ion beam re-activates transpositional acitivty of *Tto1* in tobacco or not, discuss the role of the transpositional *Tto1* played in response to the implantation of the low-energy ion beam and exposure to the vacuum.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum L.*) seeds, from selfing-line individual parent which was a cultivated variety in Henan province, were obtained from the Henan key lab of ion-beam bioengineering.

Implantation of the low-energy N^+ ion beam and investigation of the germination percentage

The tobacco seeds were implanted by low-energy (40 Kev) N^+ ion beam (generated by a machine: UIL.0.512, TNV. Russia, under a vacuum of 3×10^{-3} Pa) in four dosages:$0\times10^{17}N^+/cm^2$ under the vacuum (3×10^{-3} Pa) for 88min, 1×10^{17} N^+/cm^2, 2×10^{17} N^+/cm^2, 3×10^{17} N^+/cm^2. Three replications were done each doses, one hundred seeds were implanted each replication. The germination percentage was investigated when the seedlings had two euphyllaes as the following equation: Germination percentage=Number of the seedlings/100 seeds×100%

Extraction of the DNA

Total genomic DNA of tobacco (*N. tabacum*) was extracted, as described previously (Malone G et al. 2006), from the second euphylla of three individuals and the petals (from individual). The DNA was tested by agarose gel electrophoresis under UV light and qualified by comparing with a Low DNA Mass Ladder (Invitrogen) on 0.8% agarose gel after ethidium bromide staining.

Quantitative real-time PCR and the relative copy number of the *Tto1*

The genomic DNA were diluted 100 folds to obtain a uniform concentration of 20 ngμL^{-1}

Quantitative real-time PCR (qPCR) was performed as described in the SYBR Permix Ex TaqTM Reagent (Takara Code No DRR041S) Manual. The final volume of reaction mixture was set at 25 μL containing 2 μL DNA and 0.4mol/L forward and reverse primer. Real-time PCR were carried out in the ABI 7500 real-time PCR System (Application Biosystems, Foster, USA) with the following cycling conditions: 95 °C for 1 min, 95 °C 10 s, 56 °C 20 s and 72 °C 10 s for 40 cycles. 18s rDNA of the tobacco was used as the reference gene. The primers used as the qPCR are the following: Tot1F 5`-*GATGCAATGAGTGGTAGGTGAGATGAG*-3`; Tot1R 5`-*CTATATCTCTCTCCCTCTCAAACTCTTTC*-3`; NT18SF 5`-*GGATAACCGTAGTAATTCTA*

GAGCTAATACGT-3` ; NT18SR 5`-*AAAGTTGATAGGGCAGAAATTTGAATGATGCGT*-3` .

The relative copy number of the *Tto1* in genome was computed by the 2-△△Ct method

IRAP with the primers to *Tto1*

The amplification reaction was performed according to the protocol described by Kalendar et al (1999). One LTR primers (p*Tto1 5`-TCCGCTGTGCAGTAGTGTTTA GTG-3`*) from *Tto1* was obtained according to the described sequences in NCBI (Accession D83003). IRAP amplifications were performed in a final volume of 25 μL containing 50 ng DNA, 1U Taq DNA polymerase (Invitrogen), and 25 pmol of IRAP primer and other container. Amplification was performed in a thermal cycler model 480 (Perkin Elmer, Pomona, CA, USA) device in 0.2-mL microtubs. The amplification program consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles composed of 94°C for 30 s, 60°C for 30 s, and 72°C for 120 s for denaturation, annealing, and extension, respectively. After amplification, a final extension step was performed at 72°C for 8 min. The amplification product was separated in polyacrylamide gel and silver-stained, as described by Briard et al (2000).

Clustering

Clustering was performed by the software UPGMA clustering method with the software NTSYSpc2.1 (Numerical Taxonomy and Multivariate Analysis System) according the banding pattern generated by IRAP amplification with primers to *Tto1*, which the DNA template was from the petals.

Results

Germination percentage

The germination percentage was investigated when the seedlings had two euphyllaes. The details was listed in the table 1.

Table 1. Germination percentage of the seeds implanted by low-energy ion beam in different dosages

Samples	Controls	Vacuum	1×10^{17} (N^+/cm^2)	2×10^{17} (N^+/cm^2)	3×10^{17} (N^+/cm^2)
Germination percentage (Mean±STD)	70.33±2.52	78.0±2.0	82.33±1.53*	2±1.0**	0.67±0.58**

*and ** account for the significant level P<0.05 and P<0.01 respectively, STD: Standard Error.

Under the implantation of the dosage: 3×10^{17} N^+/cm^2, only two seeds germinated, but these two seedlings were not survived after three euphyllaes, it suggested that this dose is damaged seriously to the embryo in tobacco seed. Under the implantation of the dosage: 1×10^{17} N^+/cm^2 and exposure to the vacuum, the germination percentage was higher significantly than the controls, it suggested small-dose implantation of ion promote the germination capacity of the tobacco seeds.

New insertion events implied by the Banding pattern of IRAP

Total genomic mixed DNA pools from the second euphylla were used as the template of the IRAP, which each DNA pool was extracted from mixtures of isometric three individual euphylla. Banding pattern (Fig1 and Fig2), generated by IRAP amplification with primers to retrotransposon *Tto1*, showed that two DNA pools amplified the polymorphism bands. One is the DNA pool is exposure to the vacuum (Fig1 line 14), which had two diversity bands (indicated by arrowheads).The other is the sample treated with the dosage: 1×10^{17} N^+/cm^2 (Fig2 line 13), which had two diversity bands (indicated by arrowheads). Among these 24 DNA pools representing 72 individuals (24×3) treated by vacuum and N^+ ion, only two DNA pools did amplified the polymorphism bands, it suggested that the insertion events have a low occurrence ratio under the vacuum and N^+ ion implantation. There was no band polymorphism observed in the samples treated with 2×10^{17} N^+/cm^2 because of few survivals.

Relative copy number of *Tto1* in the second euphylla of the treated tobaccos

Copy number of the *Tto1* in the second euphylla cell were determined by relative qPCR, 18s rDNA in tobacco was used as the reference gene. The copy number of the *Tto1* was increased about 10 folds in two samples (Tabe2 showing partial results), one (Vac8 as the following) is exposure to vacuum (Va8 in Fig1), one (Ion9 as the following) is implanted by the 1×10^{17} N^+/cm^2 (Fig2 in I-9). These do not necessarily mean that the copy number was increased 10-fold in all individuals of the DNA pool, because the DNA was isolated from mixtures of three individuals. Most of the samples have isometric copy number to the controls. The mean of the copy number in the samples under the vacuum except the Vac8, is 1.15±0.22 folds, which score is from 0.88-1.29 folds. But the mean of the copy number in the samples under the vacuum including the Vac8, is 2.04±2.83 folds. The mean of the copy number in the samples implanted by implantation of the 1×10^{17} N^+/cm^2 expect the Ion9, is 1.17±0.30 folds, which score is from 0.80-1.49 fold. But the mean of the copy number in samples exposure to implantation of the 1×10^{17} N^+/cm^2 including the Ion9, is 1.80±2.38 folds. Here, a note is that the copy number could be regard as variance distinctly when the value of $2^{-\Delta\Delta CT}$ was necessarily beyond 2.0 or less than 1/2.

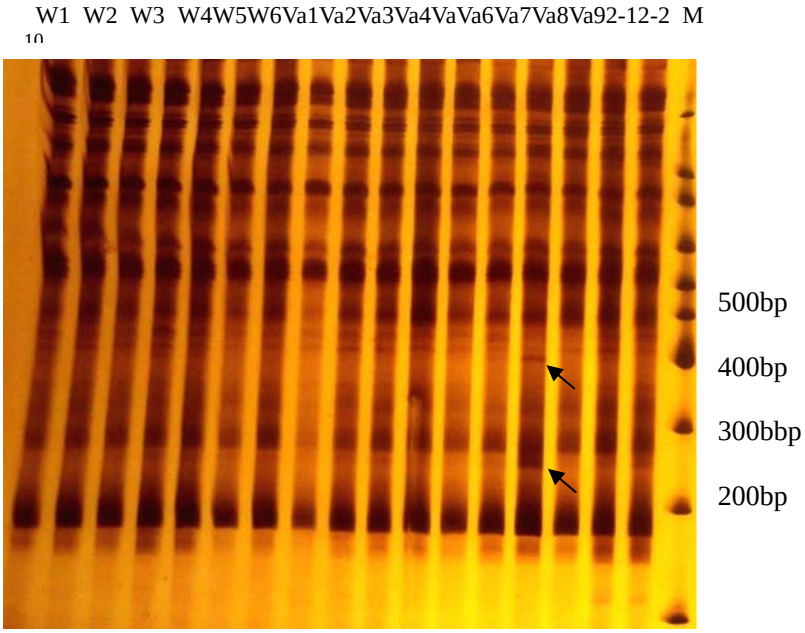


Figure1. Banding pattern generated by IRAP with primers to retrotransposon *Tto1*. Lanes from the 1-6 are the controls, including 18 individuals; Lanes from 7-15 are the samples is exposure to the vacuum, including 27 individuals; Lanes from 16-17 are the samples implanted by the ion beam with dosage: 2×10^{17} N^{+}/cm^{2}, including 6 individuals; lane 18 are the 100 bp ladder DNA marker. Marker sizes in bp are indicated on the right axis. W: the control; Va:vacuum; 2: 2×10^{17} N^{+}/cm^{2}

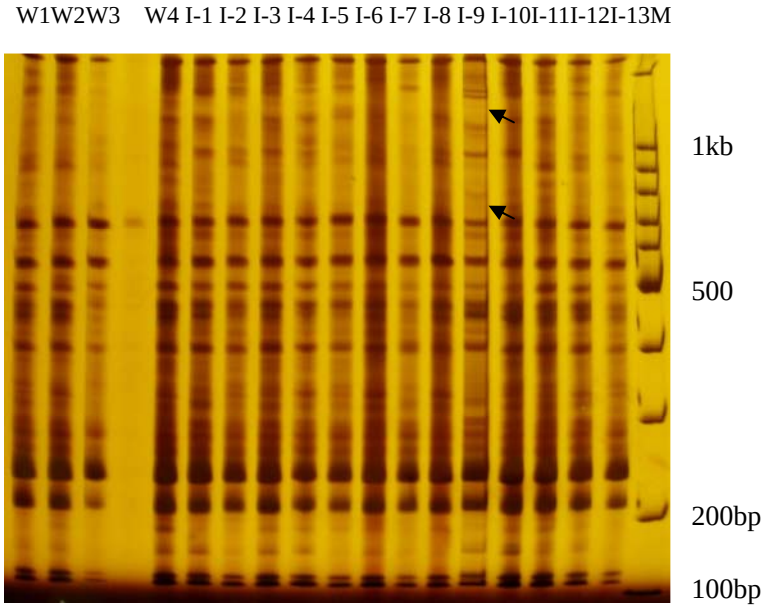


Figure 2. Banding pattern generated by IRAP with primers to retrotransposon *Tto1*. Lanes from the 1-5 are the controls, including 12 individuals; Lanes from6-18 are the samples treated by the ion beam with dosage: 1×10^{17} N^{+}/cm^{2}, including 39 individuals; lane 19 are the 100 bp ladder DNA marker. Marker sizes in bp are indicated on the right axis. W: WT or the control; I(ion): 1×10^{17} N^{+}/cm^{2}

Table2 Cycle threshold (CT) and relative copy number of *Tto1* in five samples

Samples	Average CT of 18s rDNA	Average CT of *Tto1*	△△CT	$2^{-\Delta\Delta CT}$ (Relative copy number)
Control	14.48± 0.23	14.24±0.34	----	----
Vac3	14.58± 0.13	14.12± 0.03	-0.22	1.16
Vac8	18.47± 0.42	14.90± 0.13	-3.33	10.06
Ion1	15.64±0.61	14.28± 0.01	-0.12	1.09
Ion9	17.53± 0.03	14.35± 0.04	-3.31	9.92

Cycle threshold (CT) and relative copy number of *Tto1* of five individuals were listed in this table, including the two samples which relative copy number were increased about 10 folds and two samples which relative copy number were isometric to the controls. The rows of the CT were expressed as Mean±STD

Relative copy number of *Tto1* in the petal cell

To test the idea that whether the relative copy number of *Tto1* were increased in the petal of the individuals isolated from the samples Vac8 and Ion9, which the copy number of the *Tto1* was increased 10 folds in the second euphylla. Genomic DNAs of 27 individuals, isolated from the above DNA pools:Vac8 and Ion9 , other six DNA pools selected randomly and one DNA pool of the controls, were to be amplified with IRAP protocol. The results (details not shown) showed that the mean of the relative copy number of *Tto1* in petal is 1.28±0.48 folds, and the score of it is 2.32~ 0.70 folds. However, the relative copy number of *Tto1* in petal cell of the Vac8-3, Ion9-1, Ion9-3 are increased distinctly beyond 2.0 folds (Talbe3 listed only the relative copy number of *Tto1* in the six tobacco individuals isolated from Vac8 and Ion9), that in other samples are increased slightly (1.57~0.70 folds)

Table3 CT and relative copy number of *Tto1* of the six individual petal cells which increased 10 fold in euphylla

Samples	Average CT of 18s rDNA	Average CT of *Tto1*	△△CT	$2^{-\Delta\Delta CT}$ (Relative copy number)
Control	14.74± 0.52	17.06±0.14	----	----
Vac8-1	15.13± 0.05	17.46± 0.01	0.02	0.99
Vac8-2	14.72± 0.41	17.54± 0.37	0.50	0.70
Vac8-3*	15.96±0.87	17.15± 0.23	-1.13	2.19
Ion9-1*	16.94±0.11	18.07±0.09	-1.20	2.29
Ion9-2	14.52±0.23	16.69±0.36	-0.15	1.11
Ion9-3*	15.68±0.07	16.78±0.26	-1.21	2.32

CT and relative copy number of *Tto1* of six individuals were listed in this table, including the two DNA pools which relative copy number of *Tto1* were increased about 10 fold. The rows of the CT were expressed as Mean±STD, Vac8-1, Vac8-2, Vac8-3 were isolated from the DNA pool: Vac8, Ion9-1, Ion9-2, Ion9-3 were isolated from the DNA pool: Ion9

UPGMA clustering analysis based on profile of IRAP with primers to *Tto1* in petals

The DNAs from petal of twelve individuals were amplified with IRAP protocol. UPGMA （Unweighted Pair Group Method with Arithmetic Mean）clustering analysis (Fig.3) was performed by the software NTSYSpc 2.1 according to the banding pattern (data not shown) generated by IRAP with primers to retrotransposon *Tto1* in petals. Coefficient =0.77 is considered as the demarcation of the independent groups in general. The results (Fig.3) showed that the population is classified into two groups. Ion9-3 (Relative copy number of *Tto1* increased 2.3 folds in petals) comprised an independent group for its average dissimilarity coefficient against the controls is 1.0>0.77. The rest of the eleven individuals comprised a group that is isometric to the controls, but the average dissimilarity coefficients of the Vac8-3 (Relative copy number increased 2.19 folds in petal) and Ion9-1(Relative copy number increased 2.29 folds in petal) against that of controls are 0.7 and 0.6 respectively, being closed to 0.77

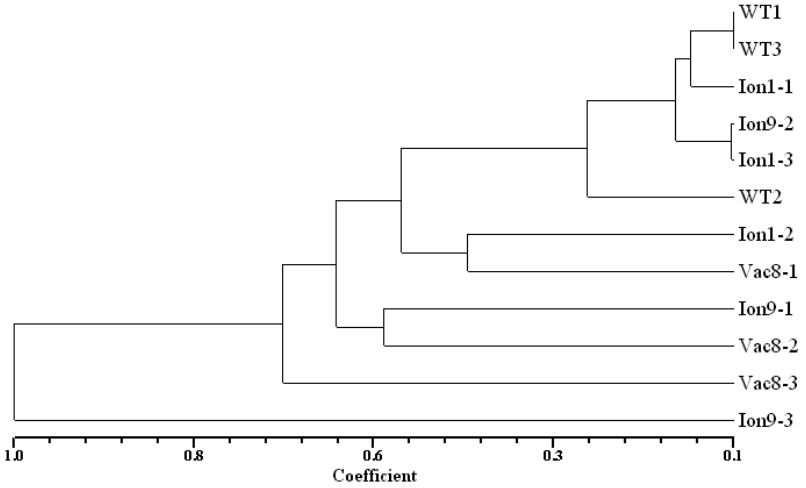


Figure 3.The clustering of the twelve individuals based on the IRAP marker analysis using the UPGMA clustering method. Coefficient is represented the average dissimilarity between individuals. WT: the controls, Ion1-1, Ion1-2, Ion-3 are the samples which *Tto1* relative copy number in genome is isometric to the controls. Vac8-1, Vac 8-2, Vac 8-3 are isolated from the DNA pool Vac8 that *Tto1* relative copy number in genome is creased to 10 folds in the second euphylla and 2.19 folds in petals. Ion9-1, Ion9-2, Ion9-3 are isolated from the DNA pool Ion9 that *Tto1* relative copy number in genome is creased to 10 folds in the second euphylla and 2.32 fold in petals.

Discussion

The implantation of ion activated the transposition of the *Tto1* in a lower frequency

Transposable elements constitute a very large proportion of eukaryotic genomes. The activation strategy of retrotransposons offers the potential for explosive increases in copy number and leads to a concomitant genome size increase (Casacuberta JM et al. 2003).The tobacco retrotransposon *Tto1*, one of a few active retrotransposons of plants, has been shown to be activated by wounding stress, exogenous supply of methyl jasmonate (Shin T et al. 1998). The copy number of *Ttol* increased 10 folds in established tissue culture cell lines; it also increased in plants regenerated from tissue cultures and in transgenic plants (Hirohiko H et al. 1993). The relative copy number of the *Tto1* in tobacco treated by vacuum and implantation of ion beam increased about 10 folds (Table2) in the second euphylla from plantlet and increased 2 folds (Table3) in petal cell from the apical meristem. On the other hand, low-energy ion beam and vacuum caused the genetic polymorphism of the *Tto1* in tobacco detected by IRAP (Fig1 and Fig2) that can be used to detect any insertion event. Furthermore, the analysis of the UPGMA clustering method based on the IRAP of the *Tto1* from the petal showed that three tobacco individuals under the vacuum and implantation of ion had serious dissimilarity to the controls. These results indicate that *Ttol* is activated by the implantation of ion and vacuum.

Although the transposition of the *Tto1* was detected in euphyllas and petals (Incidence is 3/72=0.04), this does not necessarily mean that the transposition of *Tto1* happened in all individuals treated with the implantation of the ion beam. The fact is that the insertion events of the *Tto1* occurred in a low occurrence ratio under the vacuum and low-energy ion implantation. Many retrotransposons within the genome are integrated in either a transcriptionally or transpositionally incompetent state (Vicient CM et al. 2002). Developmental or physiological factors may be required for the strong response to *Tto1* transcription to wounding stimuli. Experiments with transgenic tobacco plants carrying the *Tto1*-LTR:-glucuronidase fusion gene (LTR:GUS) revealed that *Tto1* actually contains cis-regulatory regions in response to wounding and methyl jasmonate (Shin T et al. 1998). These findings suggest that the transpositional activation of the *Tto1* is induced by suitable signals that we knew little. We think that there were few tobacco seeds having the suitable physiological state for the re-activation of the *Tto1* induced by vacuum and radiation of the ion. The transposition occurred only in the sensitive cell to the radiation, so it is not strange that transposition of the *Tto1* take place in the few tobacco individual under the vacuum and implantation of ion.

Transposition of the retrotransposons *Tto1* is possibly related to the repair of the DNA damage damaged by the low-energy ion

The transposons altered gene expression in ways that allowed the cells to respond to stress (Hirotaka EHL et al. 2007). LINE-1 elements are retrotransposons that comprise 17% of the human genome. The relationship between stress and transposition was recently exemplified by the response of LINE-1 retrotransposons to telomere damage in CHO (Chinese hamster ovary) cells (Morrish TA et al. 2007). When telomere function is disrupted by a deficiency in DNA-dependent protein kinase, 30% of LINE-1 transposition events integrate into telomere sequences. These results indicate that LINE-1 transposition is capable of repairing telomere specific DNA damage (Morrish TA et al. 2003). Retroelements can repair the genomic double-strand breaks by homologous recombination in the plant genome (Ralph S and holger P. 2002). These reports indicate that the transposition of the retrotransposons is related to the repair of the damage to the DNA molecules. Low-energy ions can reach the nuclei and cause damage to the DNA molecules, and then induce mutations when the DNA repair fails (Ya HY et al. 2007). Up to this investigation, we consider that the transposition of the retrotransposons *Tto1* is related to the repair of the DNA damage caused by the vacuum and the implantation of the ion.

At last, the conclusion is that *Tto1* is re-activated by the implantation of the ion. It implied that *Tto1* play important roles in response to the radiation of the ion. These results are helpful to fully understand mechanism of the deleted and inserted DNA of the chromosomes in cell after implantation of the ion.

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The hepatoprotective effect of dimethyl 4,4- dimethoxy 5,6,5,6- dimethylene dioxy-biphenyl- dicarboxylate (D.D.B.) on aflatoxin B1 induced liver injury

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Abstract

Seventy five samples of frozen meat, raw milk and poultry feed (25 samples each), were examined mycologically and for detection of aflatoxin B_1 (AFB_1). The results revealed that the isolated fungi represented 6 genera of moulds. The most prevalent fungi in these samples was the genus aspergillus (60%, 60% and 76%) with mean of count of ($1.6 \times 10^2 \pm 0.1$, $6.0 \times 10 \pm 0.23$ and $3 \times 10^2 \pm 1.0$), respectively, which was at the top of all isolated fungi . However, *A. flavus* was isolated form all kind of samples and that which isolated from feed produced aflatoxin B_1 with mean level of (60 ± 0.1 ppb) followed by that isolated from frozen meat (9.5 ± 0.71 ppb), but those isolated from milk had the lowest AFB_1 level (1.0 ± 0.1 ppb). The effect of dimethyl 4, 4- dimethoxy 5, 6, 5, 6- dimethylene dioxybiphenyl 2, 2- dicarboxylate (D.D.B.) in degradation of AF was evaluated by intraperitoneal injection of 30 rats with 1.5 ppm of AFB_1 to evaluate their effect on haematological, biochemical and protein electrophoretic patterns of aflatocicated rats. The obtained results indicates an improvement in the haematological picture (Hb, RBCs and PCV) together with WBCs and differential leucocytic count of the treated rats compared with non treated ones. Also, biochemical analysis revealed significant changes in urea and creatinine levels; AST and ALT activities; total protein and protein electrophoretic patterns of treated rats. The administrated of DDB effectively improved haematological alterations and prevent serum biochemical changes, ameliorated, the toxic effect of aflatoxin B1 and had hepatoprotective effect on AFB_1 induced liver toxicity. [Life Science Journal 2010;7(3):148-153]. (ISSN: 1097-8135).

Keywords: frozen meat; raw milk; poultry feed; aflatoxin B_1 (AFB_1); genus aspergillus; toxicity

INTRODUCTION

The increased population in the world requires a parallel raise in the production of food. Some countries as Egypt had to import many food and feeds. The recent researches reported that the majority of this food may carry the dangerous factors for human and animal health. Fungal contaminations and their toxins represents the most significant contaminant of these food **(Magnoli *et al.*, 1999)**. Aflatoxins are a group of secondary metabolites produced by *A. flavus* and *A. parasiticus* in food and feed commodities (**Oguz, 1997**). The consumption of food contaminated with mould and their toxins induced food poisoning, hemorrhages, hepatotoxicity, nephrotoxicity, neurotoxicity, dermatitis, carcinogenic, hormonal and immunosppression effects **(Hassan *et al.*, 2004 and 2005)**. Therefore, the degradation of such fungi and their toxins become critical demand.

It was investigated that aflatoxin B1 is the most potent one of aflatoxins (**Hamdy et al., 1995**). The dimethyl diphenyl bicarboxylate (DDB) could directly protect hepotocyte DNA from oxidative damage and inhibit TNF- alpha mRNA expression in liver tissue which resulted in prevention of liver damage (**Gao *et al.*, 2005**). Also **Park *et al.* (2005)** demonstrated that DDB exerted protection of liver from chemical- induced injury potentiated by the condition of glutathione (GSH) deficiency and has additional advantages in lowering the plasma lipids.

Therefore, this study was undertaken to screen poultry feeds, frozen meal and raw milk for A. flavus and Aflatoxin B_1 production by isolated *A. flavus* and to evaluate the effect of DDB in recovering aflatoxicosis.

MATERIALS AND METHODS

2.1. Material:

2.1.1. Samples: 75 samples of frozen meat , raw milk and poultry feed (25 of each) were collected from markets at Cairo Governorate and poultry farms for investigation of fungal contamination and detection of aflatoxin contamination.

2.1.2. Aflatoxin standard: standard of aflatoxins B_1, was purchased from sigma chemical company (USA).

2.1.3. Animals: Thirty apparently healthy albino rats weighted (100-120 g) were housed under hygienic conventional conditions in suspended stainless steel cages. Prior to experiment rats fed on healthy basal diet free from any cause of disease. Drinking water was supplied in glass bottles, cleaned three times a week.

2.1.4. Dimethyl diphenyl bicarboxylate (DDB): It was imported by Al-Ahram Pharmaceutical and Medical Equipment Company, Egypt.

2.1.5. Chemicals and reagents for using polyacrylamide gel electrophoresis : They included acrylamide, bisacrlamide, 2,2,2,2-Tetramethylethylendiamine(TEMED), B-mercaptoethanol, 1.5 M Tris-cl p H 8,8, 10% SDS(sodium dodecyl sulphate), initiator(10% ammonium persulphate), buffers, comassie stain and destaining by methanol ands acetic acid solutions, and protein

molecular weight marker. These chemicals were purchased from sigma chemical company , USA.

2.2. Methods:

2.2.1. Isolation and identification of moulds: each feed samples was subjected for isolation and identification of fungi as recommended by (**Conner *et al.*, 1992**).

2.2.2. Production and estimation of aflatoxins (**Gabal *et al.*, 1994**).

2.2.2.1. Cultivation and extraction of aflatoxins:

Isolated strains of *Aspergillus flavus* were inoculated into flasks conaining 50 ml of sterile yeast extract solution 2% containing 20% sucrose (YES).

Inoculated flasks were incubated at 25ºC for 7-10 days . At the end of the incubation period, 50 ml chloroform were added and the mixture was thoroughly mixed for one minute in ultraurax apparatus, then centrifuged (3000 r.p.m.) for 10 minutes after which the chloroform layer decanted. The chloroform extraction was repeated for one more time.

One ml ethanol, 3 gm copper – (III)-hydroxide carbonate and 5 gm anhydrous sodium sulphate were added to the chloroform extract, mixed well and filtered.

The filtrate was then evaporated in a rotatory vacuum evaporator, the residue cooled and resuspended in exactly 5 ml of colorophorm.

2.2.2.2. Thin layer Chromatographic analysis of choloroform extract (Scott, 1990);

The concentrated extract was spotted onto activated thin layer chromatography plates coated with silica gel of 0.25 mm thickness. Standard solution of aflatoxins B_1, B_2, $G_1$and G_2 were spotted on the plate using 10-20 µl capacity pipette. The spotes were air dried and the TLC plates out in the developing tank containing the developing solvent system (5Toluene :4 ethyl acetate :1 of 90% formic acid (V/V/V) or (chloroform: hexan: petroleum ether: benzene: acetone 6:1:1:1:1).

When the solvent travels about 12 cm front, the plates were removed from the tank, air dried and inspected under a ultraviolet light lamp for examining the tested and standerd spots and determining the rate of flow (R_f[1]of the toxin) then the results recorded

$$R_f = \frac{\text{Distance traveled by unknown material}}{\text{Distance traveled by solvent front}}$$

Aflatoxin was calculated by the following equation or formula.

$$\mu g/kg = \frac{S \times Y \times V}{Z \times W}$$

S = µl of aflatoxin standard equal to unknown.
Y = Concentration of aflatoxin standard in µg/ml
V = µl of final dilution of sample extract
Z = µl of sample extract giving a spot fluorescent intensity equal to the standard (S)
W = Mass of sample, represented by the final extract in gm. Applied to Column =(100 g x filtrate volume)/ 200.

2.2.3. Experimental design: Thirty rats were divided into 3 equal groups. Rats of the first group were given normal feed (free from mycotoxins and without any treatment) and kept as a negative control. Rats of the other two groups were given single dose of AFB_1 intraperitonealy at the rate of 1.5 ppm **(Bao, 2002)**. Then on the second day rats of the third were dosed orally by 300 mg DDB for 3 weeks **(Bao, 2002)**, while those of the second group were left without any treatment to kept as positive control.

2.2.4. Haematological and biochemical investigations:

At the end of the experimental period, two blood samples were collected from each rat. The first portion was collected in small labeled dry and clean vials containing Na EDTA (1 mg/ 1ml fresh blood, **Schalm *et al.*, 1975)** as anticoagulant for haematological study according to routine methods described by **Jain (1986)**. While, the second portion was taken without anticoagulant in centrifuge tube, allowed to clot, then centrifuged at 3000 rpm for 10 minutes for separation of serum which used to assay the biochemical parameters. Serum analysis included estimation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities according to **Reitman and Frankel, (1957**), serum urea according to **Wybenga *et al.* (1971**), serum creatinine level according to **Henry (1974) and** total serum protein as described by **Pesce and Kaplan (1978**).

2.2.5. **Estimation of molecular weight of different plasma protein by using SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970):** After the separating gel was prepared and pored into wells , rinse with distilled water and invert to drain the wells. 25 ul of serum sample underlay in each well and the upper buffer chamber putted in place . Then , contact sandwiches to the bottom of the upper chamber .The upper buffer chamber was placed on the heat exchanger in the lower buffer chamber . Fill the lower buffer chamber with tank buffer until the sandwiches are immersed in buffer and add a drop of 0.1 % phenol red to the upper buffer chamber (tracking dye). Alternatively, add the dye directly to the sample after it has been heat-treated. The upper buffer chamber was filled with tank buffer .After placing the lid on unit, the power supply was connected(PS 500XT). The cathode should be connected to the upper buffer chamber . Turn the power supply on and adjust the current 2 m. Amp. Per sample for 90 minutes, the voltage should start at about 70-80 V, but will increase during the run. When the dye reaches the bottom, turn the power supply off and disconnect the power cables. The gels sandwiches placed in stain and gently shake the gels for one hour on the PR 70 red rotor. Then put it in destaining solution and shake for one hour. Quantitation of different protein molecular weight were made by using densitometer G 700 (Bio-Rad, USA).

2.2.6. Estimation of isoelectric focusing of plasma protein by using polyacrylamide gel electrophoresis as described by O'Farrell (1975):

The isoelectric focusing is carried out with anode electrode solution (0.01 M 3PO4) and cathode electrode solution (0.02 M Na OH) which are laid along the long length of each side of the gel and a potential difference applied. Under the effect of this potential difference, the ampholytes from a PH gradient between the anode and cathode, (the gels are pre-run at 200 V for 15 minutes at 22 °C and 400 V for 30 min at 22 °C). Depending on which point on the PH gradient the sample has been loaded, protein that initially at a PH region below their isoelectric point will be positively charged and will initially migrate towards the cathode. As they proceed, however, the surrounding PH will be steadily increasing and therefore the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point zwitterions form with no net charge. Likewise substances that are initially at PH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric point and become stationary. The gels are fixed in 50% v/v ethanol, 7% v/v acetic acid for 2 hours. The fixation gel must be done before staining because the ampholytes will stain too, giving a totally blue gel, the fixation will precipitate the proteins in gel and allows the much smaller ampholytes to be washed out. After staining and destaining of gels, the distance of each band from one electrode is measured and graph of distance for each protein is determined using densitometer G 700 (Bio-Rad, USA).

2.3. Statistical analysis: The obtained date were computerized and analyzed for significance. Calculation of standard error and variance according to (**SPSS 14, 2006**).

RESULTS AND DISCUSSION

The current data in table (1) showed isolation of 6 genera of mould and one yeast species. The most prevalent fungi in frozen meat, raw milk and poultry feed was the genus aspergillus (60%, 60% and 76%) with mean of count of ($1.6 \times 10^2 \pm 0.1$, $6.0 \times 10 \pm 0.23$ and $3 \times 10^2 \pm 1.0$), respectively, which was at the top of all isolated fungi. Other genera of mould were recovered in different frequency. Whereas, the yeasts were isolated in higher frequency (44% with the mean count of ($3.5 \times 10 \pm 0.1$ in frozen meat) (40% with the mean count of $1.1 \times 10^2 \pm 0.25$ in raw milk) and (20% with mean count of $4 \times 10^2 \pm 0.3$) in poultry feed.

Aspergillus flavus was the most frequent mould of aspergillus species isolated from all tested samples of frozen meat, milk and poultry feed (44% with mean count of $1.6 \times 10 \pm 0.3$), (60% with mean count of $3.3 \times 10 \pm 0.3$) and (60% with mean count of $1.0 \times 10^2 \pm 0.2$) respectively. The similar results were previously reported by **Hassan *et al.* (1997); Wafia and Hassan (2000); Hassan and Hamad (2001); Hassan *et al.*, (2002, 2004 and 2005)** and **El- Ahl *et al.* (2006)**.

Other members of aspergillus were isolated in various frequency (Table, 2). The isolation of large numbers of fungi in present samples may be due to their exposure to environmental factors as high temperatures and humidity during harvesting, transportation, handling, processing and/or storage which help in all ways to fungal pollution by different genera of fungi. Significant levels of aflatoxin was produced by *A. flavus* isolated from present samples (Table, 3), where, the maximum levels of toxin was obtained from *A. flavus* isolated from poultry feed (80% of isolates produced mean level of 60 ± 0.1 ppb) followed by those isolated from frozen meat (50% with the mean level of 9.5 ± 0.7 ppb). However, *A. flavus* recovered from raw milk showed the lowest rat of toxin production (30%) with mean level of (1.0 ± 0.1 ppb).

Results of haematological study as presented in table (4) showed significant reduction in haemoglobin level (Hb), red blood cells (RBCs) and Packed cell value (PCV) of rats given aflatoxin only (group 2) indicating presence of anaemia. This anaemia may be due to the direct effect of the toxin on the haemobiotic system. Similar findings were reported by **Hassan and Mogda (2003)** and **Salem *et al.* (2007)**.

The treatment with DDB significantly improved the function of this system by degradation of aflatoxin and It improved concentration of Hb, RBCs count and haematocrit value**(Abdel- Hameid (2007)**.

Results presented in table (5) showed significant leucocytosis in groups 2, 3 compared to group 1, this recorded leucocytosis may be attributed to the toxic effect of AFB_1 on haemobiotic tissue as recorded by **Parent-Massin (2004)**. It could be noticed from the tabulated results in table (5), that treatment with DDB improved the total leucocytic count compared to the aflatoxicated rats toward the control –ve group (group, 1). The results in table (5) revealed a significant increase of segmented and staff cells percent while lymphocytes, monocyte, eosinophils and basophils were lowed in group 2 which received AFB_1 only without any treatment compared to the control –ve group. On the other hand treatment with DDB induced an improvement in segmented, lymphocytes, monocyte, basophils and staff percent compared to aflatoxicated group toward the control – ve group.

Concerning the effect of AFB_1 alone on the kidney and liver functions of rats, a significant elevation in levels of urea, creatinine, AST and ALT activity were observed. **Aravind *et al.* (2003)** reported that AFB1 caused an increase in AST and ALT activities, which indicated the liver damage. Also, **Bilgic and Yebyldere (1998)** reported that AFB_1 cause petecial haemorrhages in the kidney and liver due to the animals feeding on diets containing AFB_1. This findings was in agreement with results obtained by **Celyk *et al.* (1999)** and **Eraslan *et al.* (2006)**. The treatment with DDB leads to a decrease in AST and ALT activities due to protection of treated rats against AFB_1 hepatotoxicity by increasing the detoxifying metabolism of AFB_1 in the liver as recorded by **Lu and Li (2002)**. Similar results were reported by **Gao *et al.* (2005)** recording that DDB significantly inhibited hepatocyte nuclear DNA fragmentation and prevented the direct DNA damage, these results suggest that DDB could directly protect hepatocyte DNA from oxidative damage and inhibit tumor necrosis factor (TNF)- alpha

mRNA expression in liver tissue, which resulted in prevention of liver damage. Similarly, **Park *et al.* (2005); Sun and Lu (2006)** and **Jin *et al.* (2007)** recorded that DDB effectively prevented increases in plasma transferases. **Abdel- Salam *et al.* (2007)** and **Abdel-Hameid (2007)** reported that DDB has significantly prevented the occurrence of liver damage. On the other hand this findings disagreed with that reported by **Kin *et al.* (1999)** and **Nan *et al.* (2000)** who recorded that DDB did not improve AST and ALT activities caused by hepatotoxicity. Results shown in table (7) indicated that the effect of DDB on the total protein and its electrophoretic pattern in aflatoxicated rats showed a significant improvement in their serum levels. This elevation may be attributed to the improvement in hepatocytes as reported by **Lu and Li (2002)** leading to enhancement of protein synthesis which was impaired by aflatoxicosis. The reported impairment of protein synthesis due to aflatoxicosis was in agreement with that reported by **Raju and Devegawda (2000); Aravind *et al.* (2003); Don and Kaysen (2004)** and **Eraslan *et al.* (2006)**.

The presence of fungi and their toxins in feed and food reflected unhygienic measure during cultivation, irrigation harvesting transportation, handling, storage and processing of feed and food. Therefore, frequent testing programs of food during different steps of production must be monitored before given to animals or human for consumption. The fungal inhibitors may be added if the level of contamination over the limited level. Therefore, continuous investigations for finding new safe methods for controlling the growth of fungi and mycotoxins production to keep the human and animals consumer are critical demand. All ways for increasing the quality of human health and animals wealth.

Table (1): Mycoflora of frozen meat, raw milk and poultry food.

Fungal genera	Prevalence of fungal genera								
	Frozen meat (25)			Raw milk (25)			Poultry feed (25)		
	No. of +ve	%	Mean of count ± SE	No. of +ve	%	Mean of count ± SE	No. of +ve	%	Mean of count ± SE
Total fungi	20/ 25	80	$3.8 \times 10^2 \pm 2.0 \times 10$	17/ 25	75	$6.1 \times 10 \pm 0.2$	22/ 25	88	$3 \times 10^2 \pm 1.0 \times 10$
Aspergillus sp.	15/ 25	60	$1.6 \times 10 \pm 0.1 \times 10$	15/ 25	60	$6.0 \times 10 \pm 0.23$	19/ 25	76	$3 \times 10^2 \pm 1.0 \times 10$
Penicillium sp.	8/ 25	32	$1.1 \times 10 \pm 0.3 \times 10$	12/ 25	48	$1.8 \times 10 \pm 0.25$	15/ 29	60	$4x\ 10^1 \pm 0.7x10$
Fusarium sp.	5/ 25	20	$1.5 \times 10 \pm 0.2 \times 10$	1/ 25	4	$0.5 \times 10 \pm 0.0$	10/ 25	40	$5 \times 10 \pm 2x10$
Cladosparium sp.	9/ 25	36	$3.5 \times 10 \pm 0.1 \times 10$	10/ 25	40	$1.1 \times 10 \pm 0.2$	25	20	$4 \times 10^2 \pm 0.3x10$
Mucor sp.	6/ 25	24	$2.1 \times 10 \pm 0.1 \times 10$	5/ 25	20	$1 \times 10 \pm 0.12$	10	40	$0.3 \times 10^1 \pm 2.0x10$
Rhizop sp.	4/ 25	16	$1.0 \times 10 \pm 0.1 \times 10$	4/ 25	16	$0.5 \times 10 \pm 0.2$	6	20	$1x10^2 \pm 0.2x10$
Yeast sp.	11/ 25	44	$2.0 \times 10 \pm 0.1$	5/ 25	20	$1 \times 10 \pm 0.3$	5	20	$10^1 \pm {}^{0.03x10-}$

25 samples were examined.

Table (2): prevalence of members of Aspergillus spp. in frozen meat, milk and poultry feed.

Fungal genera	Prevalence of fungi								
	Frozen meat			Raw Milk			Poultry Feed		
	+ve	%	Colony count ± SE	+ve	%	Colony count ± SE	+ve	%	Colony count ± SE
A. flavus	11	44	$1.6 \times 10 \pm 0.2x10$	15	60	$3.3 \times 10 \pm 0.3x10$	15	60	$1 \times 10^2 \pm 2x10$
A. niger	10	40	$2.8 \times 10 \pm 0.3x10$	12	48	$2.8x\ 10 \pm 0.2x10$	10	40	$2 \times 10 \pm 1x10$
A. candidus	8	32	$2.5\ x10 \pm 0.1\ x10$	3	12	$1x10 \pm 0.2x10$	8	32	$0.5x10^2 \pm 0.03$
A. fumigatus	6	24	$1.0 \times 10 \pm 0.0$	8	32	$1.6x10 \pm 0.2x10$	7	28	$3x10 \pm 0.1x10$
A. ochraceus	3	12	$0.7 \times 10 \pm 0.0$	4	16	$1x10 \pm 0.1x10$	13	52	$1x10^1 \pm 0.3x10$
A. terrus	3	12	$1 \times 10 \pm 0.0$	2	8	$1.3x10 \pm 0.1x10$	5	20	$0.5x10 \pm 0.0$

25 samples were exam.

Table (3): Rates of aflatoxins production by *A. flavus* isolated from frozen meat, milk and poultry ration.

Source of isolates	No. of isolates	+ ve samples		Mean of count	Levels of AF ppb		
		No.	%		Max	Min	Mean ± SE
Frozen meat	10	5	50	16 ± 2.0	14	5.5	9.5 ± 0.71
Raw Milk	10	3	30	10 ± 2.0	2.0	0.5	1.0 ± 0.1
Poultry feed	10	8	80	10 ± 0.042	1000	150	60 ± 0.1

Table (4): Haematological picture of aflatoxicated rats and those treated with DDB and control ones.

	Hb	RBCs	PCV
Control 1	14.6 ± 0.33^{A}	5.57 ± 0.17^{A}	42.4 ± 0.92^{A}
Group 2	12.6 ± 0.11^{aB}	4.88 ± 0.058^{aB}	38.2 ± 0.374^{aB}
Group 3	15.12 ± 0.16^{bc}	5.65 ± 0.183^{abcD}	45.6 ± 0.245^{abD}
F-calculated	28.022#	28.788#	8.377#
LSD	0.75333	1.8500	1.4333

Significant at $P < 0.05$ using ANOVA test

Aa, Bb, Cc Significantly different between two comparison groups against capital litter at $P < 0.05$ using LSD.

Group 1: control –ve

Group 2: treated with aflatoxin

Group 4: treated with aflatoxin + DDB

Table (5): Total and differential leucocytic count of aflatoxicated rats and those treated with DDB and control ones.

	WBCs X 10^3/mm^3	Differential lymphocytic count					
		Segment	Lymphocytes	Monocyte	Eosinophils	Basophils	Staff
Control 1	11.02 ± 0.32^{A}	30.6 ± 1.03^{A}	61.2 ± 0.73^{A}	3.8 ± 0.49	3.4 ± 0.25^{A}	0.6 ± 0.24	0.4 ± 0.24
Group 2	17.8 ± 0.192^{aB}	42.00 ± 0.447^{aB}	51.4 ± 0.4^{B}	3.2 ± 0.374	2.4 ± 0.245^{a}	0.40 ± 0.245	0.80 ± 0.20^{B}
Group 3	14.78 ± 0.107^{ab}	32.20 ± 0.20^{bD}	61.2 ± 0.489^{bD}	2.60 ± 0.245	2.80 ± 0.20^{c}	0.60 ± 0.245	0.40 ± 0.245
F-calculated	8.377#	111.558#	130.729#	2.174	4.529#	0.207	3.500#
LSD	0.68400	4.8500	5.5000	--	0.750	--	0.8000

Significant at $P < 0.05$ using ANOVA test

Aa, Bb, Cc Significantly different between two comparison groups against capital litter at $P < 0.05$ using LSD.

Group 1: Control –ve Group 2: treated with aflatoxin Group 3: treated with aflatoxin + DDB

Table (6): The induced effect of DDB treatment on urea, creatinine and tarnsaminases activities in aflatoxicated rats and control ones.

	Urea	Creatinine	ALT	AST
Gp 1	19.45 ± 0.36^{A}	0.56 ± 0.003^{A}	17.50 ± 0.47^{A}	56.0 ± 0.31^{A}
Group 2	31.86 ± 0.99^{aB}	0.712 ± 0.012^{aB}	34.2 ± 0.86^{aB}	86.6 ± 2.56^{aB}
Group 4	30.66 ± 0.546^{cD}	0.70 ± 0.007^{acD}	26.4 ± 0.812^{abD}	75.6 ± 1.50^{abcD}
F-calculated	74.981#	70.949#	98.615#	118.355#
LSD	3.091	0.024	6.916	8.266

Significant at $P < 0.05$ using ANOVA test

Aa, Bb, Cc Significantly different between two comparison groups against capital litter at $P < 0.05$ using LSD.

Group 1: Control –ve Group 2: treated with aflatoxin

Group 3: treated with aflatoxin + DDB

Table (7): Serum protein electrophoretic pattern of toxicated rats and those treated with DDB and control ones.

Groups	T.P.	Albumin	Alpha1	Alpha2a	Alpha2b	Beta1	Beta2	Gama1	Gama2a	Gama2b
Control	7.64 ± 0.64^{A}	2.08 ± 0.011^{A}	0.523 ± 0.016	0.41 ± 0.016^{A}	0.646 ± 0.018^{A}	1.217 ± 0.007^{A}	0.533 ± 0.006^{A}	1.2 ± 0.021^{A}	0.67 ± 0.019^{A}	0.353 ± 0.004^{A}
Group 2	7.11 ± 0.046^{B}	1.748 ± 0.009^{aB}	0.544 ± 0.009	0.398 ± 0.008^{B}	0.54 ± 0.004^{aB}	1.06 ± 0.01^{aB}	0.50 ± 0.007^{aB}	1.096 ± 0.012^{a}	0.834 ± 0.014^{a}	0.39 ± 0.027^{B}
Group 4	7.86 ± 0.059^{abcD}	2.09 ± 0.019^{bcD}	0.526 ± 0.009	0.498 ± 0.008^{abcD}	0.644 ± 0.008^{bcD}	1.138 ± 0.008^{abcD}	0.59 ± 0.008^{abD}	1.118 ± 0.0107^{aD}	0.870 ± 0.011^{ac}	0.388 ± 0.009^{cD}
F-calculated	53.491#	171.594#	1.089	36.273#	30.069#	63.702#	43.379#	15.345#	39.271#	12.618#
LSD	0.22133	0.08667	--	0.5333	0.04583	0.0333	0.0333	0.08200	0.05333	0.04550

Significant at $P < 0.05$ using ANOVA test

Aa, Bb, Cc Significantly different between two comparison groups against capital litter at $P < 0.05$ using LSD.

Evaluation of total proteins showed significant increase in group 4 which administrated aflatoxin and DDB together with salicylic acid.

Group 1: Control –ve - Group 2: treated with aflatoxin-Group 4: treated with aflatoxin + DDB

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