The Protective Role of Alpha Lipoic Acid Against pesticides Induced testicular toxicity. (Histopathological and Histochemical Studies)

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Abstract: The present study investigated the efficiency of alpha-lipoic acid (ALA), in ameliorating some of biochemical and histological alternations induced by intoxication with a mixture of well known pesticides for 28 days. 4 groups of rats were treated as follows G1; untreated control animals, G2 (p-mix, consists of 1/60LD50 chloropyrifos (2mg/Kg b.wt) 1/200 LD50 of fenitrothion (2.5 mg/km b.wt). G3 (ALA 200 mg/animal), G4 (p-mix+ALA). Blood samples were taken at, 14 and 28 days for further biochemical parameters and specimens of testes were subjected for histopathological, histochemical and immunohistochemical studies. In light microscopic examinations, histopathological observation of the treated rats revealed significant alterations in the testis tissue of pesticides mixture treated group including focal mild testicular damage, blood hemorrhage and hypospermatogenesis, necrosis and atrophy. Also the histological results using masson-trichrome stain revealed various fibrosis grades between the testis tissues upon the exposure to the insecticides. Immunohistochemical study, using TUNEL technique showed an increase in the incidence of positive apoptotic cells between the germ cells. Also complete depletion of the level of acid phosphatase enzyme which involve in the biosynthesis of testosterone in the testis tissue. 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 grade of fibrosis between testis tissues. The incidence of apoptotic cells level recorded back to its normal view. Conclusion: The biochemical, histopathological, immunohistological reports supported that the pesticides have many implicated changes on the testes and reproduction and the antioxidants like lipoic acid obtained many trials to get ameliorative effects on the toxicity of pesticides.


Keywords: Pesticides – Reproduction - Apaptosis - Fibrosis – Antioxidants.

Introduction

Pesticides are agricultural chemicals used for controlling pests on the plant or animals. 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). It has many structural actions of insecticides as the inhibition of the release of the acetylcholonesterase at the synaptic junction (Roy et al., 2004). Inhibition of liver acetylcholinesterase (AChE) activity is generally regarded as a useful indicator of poisoning by organophosphorous pesticides. Additionally, several studies showed that organophosphorous as malathion-induced various physiological, biochemical, immunological and histological changes in experimental animals (Tamura et al., 2001; Selvakumar et al., 2004 and Tamura et al., 2008).

The widespread use of organophosphates has stimulated research into the possible existence of effects related with their reproductive toxic activity (Pajoumand et al. 2002).

Pesticides have the potential to cause reproductive toxicity in animals, affect human reproduction (Hileman, 1994). Some pathological effects of pesticides on the reproductive system of experiment animals were recorded by many authors, (Okamura et al., 2005 and Presibella et al., 2005). Chlorpyrifos caused 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 pesticides both natural or industrial, having estrogenic property may be causing a variety of reproductive disorders in wildlife and human population (Chitra et al., 1999). However, not many studies have been conducted in animals yet (Pesch et al., 2006). Pesticides with such properties have been shown to cause over production of reactive oxygen species (ROS) in both intra and extra cellular spaces, resulting in a decline of sperm count and infertility in wildlife and human (Gangadharan et al., 2001). A significant reduction in the sialic acid
content of testes and testicular glycogen was noticed, whereas the protein and cholesterol content was raised at significant levels. All these toxic effects are moderate at low doses and become severe at higher dose levels. From the results of the other study it is concluded that chlorpyrifos induces severe testicular damage and results in reduction in sperm count and thus affect fertility. Small changes in sperm counts are known to have adverse affects on human fertility (Ibrahim and ElGamal, 2003), Chlorpyrifos (CPF) and Fenitrothion, are organophosphorous insecticides, widely used for controlling a wide range of insects and pests. It has been reported that OPs may induce oxidative stress in humans (Vidyasagar et al., 2004) and animals (Verma, 2001) when acutely exposed. On the other hand, many insecticide families as pyrethroids exhibit neurological activity and causes neurological damage, but at different target site. Several studies of varying durations of exposure with organophosphorus or pyrethroid pesticides have postulated a possible role for the generation of free radicals and induction of oxidative stress (Tuzmen et al., 2008). Oxidative damage can occur to many classes of molecules, including lipids, proteins, nucleic acids, and sugars. In a tissue like the testis has high rates of metabolism and cell replication, oxidative stress can be especially damaging, the antioxidant capacity of the tissue very important in the continuity of spermatogenesis process.

Fibrosis of atrophic tubules is frequently associated with thickened basement membranes and interstitial spaces fibrosis. Fibrosis is probably the end result of an inflammatory process; there was also a significant correlation between increased fibrosis and both reduced tubular diameter and fewer germ cells (Suskind et al., 2007).

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, apoptosis appears to play a major role during spermatogenesis, previous morphological studies have implicated apoptosis in spermatogonial death during spermatogenesis (Bartke, 1995). Death of selected spermatocytes and spermatids is also a regular feature of normal spermatogenesis and about 20% of germ cells degenerate between preleptotene primary spermatocytes and mature spermatids. Withdrawal of gonadotropins and/or testosterone enhances the germ cell degeneration and it seems that apoptosis of germ cells in the testis is under the control of FSH and testosterone (Bartke, 1995; Billig et al., 1995; Henriksen et al., 1995; Hikim et al., 1995; Kangasniemi et al., 1995). Mathew et al. (1992) reported that treatment with methyl parathion alters sperm development in mice suggesting that this OP is genotoxic for germ cells.

Lipoic acid is an organosulfur compound, which is an essential cofactor for many enzyme complexes. Naturally occurring lipoic acid is always covalently bound and not immediately available from dietary sources. Additionally, the amount of lipoic acid present is very low. Studies are generally dealing with the biological consequences of lipoic acid administration and its derivatives in cases associated with oxidative stress (Gotz et al., 1994; 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. Testicular toxicity, assessed by decreased enzymatic activities of lactate dehydrogenase and glucose-6-phosphate dehydrogenase, was reversed with lipoic acid pretreatment. CP-exposed rats showed abnormal levels of enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase and antioxidants as reduced glutathione, ascorbate and α-tocopherol) along with high malondialdehyde levels. 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 therefore, undertaken 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.

Materials & 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 °C dark/light cycle.

Chemicals

Chlortpyrifos: pyribin (chlorpyrifos 48%EC) (O,O – Diethyl-O(3,5,6-trichloro-2-pyridyl phosphorothioate) 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 incuba
tion every day for 28 days.

Group II (P-mix): rats were orally treated via gastric intubation with mixture of pesticides mixture contain (1/60LD50 chloropyrifos =2mg/Kg b.wt, 1/200 LD50 of fenitrothion =2.5 mg/kg gm b.wt every day for 28 consecutive days.

Group III (ALA): rats were orally supplemented with 60mg /Kg for 28 days and served as +ve control

Group III (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 heparinized 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° for subsequent use. At the end of the experiment, animals were dissected and samples of the testis were subjected to the histopathological and histochemical studies.

Biochemical assay

Malondialdehyde (MDA) occurs in lipid peroxidation and 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 et al., (2001).

Histopathological studies

Animals were sacrificed after 24 hour of treatment. The spleen was dissected and fixed immediately in neutral buffered formalin (10%) and paraffin sections were prepared and stained with hematoxylin and eosin. 2- Masson-trichrome was used for the qualifying collagen and elastic fibers changes. (Bancroft and Gamble, 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 testicular 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 4°C, 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 liver 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 (cytoplasmic 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 OD405.

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 liver tissue was immediately fixed in 4% paraformaldehyde at 4°C 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 37°C 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 37°C. 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 formaldehyde and acid phosphatase was detected due to Gomori lead method in which acid phosphatase activity acquire black and the nuclei green colours according to Bancroft and Stevens, 2001.

**RESULTS**

**Biochemical studies**

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.

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)

<table>
<thead>
<tr>
<th>Parameter Groups</th>
<th>Con.</th>
<th>P-Mix</th>
<th>ALA (+ ve C)</th>
<th>ALA+ P-mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/dl)</td>
<td>17.22 ± 1.77</td>
<td>31.71 ± 1.66 *</td>
<td>19.97 ± 3.57</td>
<td>21.96 ± 2.99</td>
</tr>
<tr>
<td>Testosterone (ng/dl)</td>
<td>211.699±20.11</td>
<td>143.043± 11.16 a</td>
<td>207.699 ±16.22</td>
<td>192.779 ± 19.27ab</td>
</tr>
</tbody>
</table>

All data were expressed as mean ± SE. * significance difference versus control at P<0.05, a significance difference versus control at P<0.05. b significance difference versus control at P<0.05. MAD = malondialdehyde.

**Histopathological observations**

(A)- Examination of sections of the testes with hematoxylin 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. (Fig 1). 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)( Figure 8). The testis tissue of the animals treated with (60mg/kg b.wt.) of lipoic acid showed mild fibrosis around the testis section (Figure 9). The pesticides mixture treatment obtained higher grades of fibrosis surrounding the testis section (tunica albuginea) and in the peritubular areas (Figure 10, 11). Supplementation of alpha lipoic acid after the toxicity of pesticides mixture showed testis tissues with minimal fibrosis (Figure 12).
Figure 1: Photomicrograph of testis section of untreated rat showed normal appearance of testis structure (Arrows) and seminiferous tubules (S). Figure 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). Figure 3, 4: Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifos and 1/200 LD50 of fenitrothion) showed infoldings of the membranes of seminiferous tubules, degeneration of the spermatogenesis process and hemorrhage between the deminiferous tubules (arrows).

Figure 5: Photomicrograph of testes sections of rats also treated with pesticide mixture obtained more proliferation within the interstitial cells (arrows). Figure 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)

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 (Figure 13). Upon supplementation with alpha lipoic acid alone the testis tissue revealed mild increase in the positive cells between their germ cells (Figure 14). The testis section of the animals intoxicated with pesticides mixture obtained significant highly positive cells between all the stages of spermatogenesis cells (Figure 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 (Figure 16).
Figure 8: Photomicrograph of testis section of untreated rat stained with masson trichrome stain showed the tunica albuginea normally arranged (arrows). Figure 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). Figure 10, 11: Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifos and 1/200 LD50 of fenitithione) showed high degree of fibers around the seminiferous tubules (arrows 10) and around the testis (arrows 11). Figure 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.

Figure 13: Photomicrograph of testis section tissue of untreated rat showed normal positive cells between the spermatogenesis cells (arrows). Figure 14: Photomicrograph of testes section of rat treated with (60mg/kg) alpha lipoic acid showed some positive cells within germ cells (arrows). Figure 15: Photomicrographs of testes sections of rats treated with insecticide mixture of (1/60 of LD50 of chlorpyrifos and 1/200 LD50 of fenitithione) showed significant increase in the brown positive cells within the primary stages of germ cells (arrows). Figure 16: Photomicrograph of testes sections of rats intoxicated with the pesticide mixture and treated with ALA revealed nearly decrease of the positive cells (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 (Figure 17). The animals treated with alpha-lipoic acid (served as control group) showed nearly normal level of the brown granules of acid phosphatase enzyme (Figure 18).

The animals treated with the pesticides mixture obtained complete depletion in the brown granules of acid phosphatase enzyme. (Figure 19) compared to the untreated animals. Alpha – lipoic acid treatment with the pesticides mixture showed mild ameliorative effect on the concentration of the enzyme granules (Figure 20) compared to the pesticides treated group.

Discussion

The testicular toxicity of insecticides mixtures was proven and alternative harmless control strategies should be applied. Insecticides induced sever testicular toxicity as shown in histopathological results which coupled with markedly changes of biochemical results. Our results obtained spermatogonial depletion and atrophy due to pesticides toxicaion 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 accompanied by disappearance of interstitial cells and Leydig cells.

There are several possible mechanisms for the antigonal actions of organophosphorous 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; Serim, 2007). The hazardous effect of these pesticides on semen quality continued during the post treatment period, and was dose-dependent (Cakir, 2005). Therefore Roy et al (2004) showed that quinalphos 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, it is also likely that apoptosis correlates 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). Acid phosphatase enzyme plays an important role in the process of cell metabolism, autolysis, differentiation and many related processes. (Sugar, 1997). Dilatation of blood capillaries in between seminiferous tubules is the result of acid phosphatase enzyme activity. The increase in acid phosphatase 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.1988 and Ibrahim et al., 2003).

Our results explained that the pesticides (delta methrin and chlorpyrifos) alter reproduction function with imbalance of hormonal system influenced by oxidative stress and finally the histological modifications. The depletion of the enzymatic antioxidative system strengthens the oxidative damage of membranes plays a significant role in cellular damage into the testes (Parasmith 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 when it was found it prevented the symptoms of vitamin C and vitamin E deficiency. Dihydrolipoic acid is able to regenerate (reduce) antioxidants, such as glutathione, vitamin C and vitamin E (Biewenga et al., 1995 and Packer 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. At the membrane level, you get protection as free radicals try to enter the cell. Addition of ALA into the extender media allows the antioxidant (ALA) to protect these components by creating a shield surrounding the mid-piece (aqueous layer) and within the lipid layer structure itself (Cronan et al., 2005). The ability of ALA to create a robust shield on the cell membrane, along with the liquid that surrounds the sperm indirectly enhance the ability of the sperm to tolerate higher volumes of free radical attack. Addition of ALA is thought to have assisted in the metabolism of oxidative deacboxylation by acting as a co-enzyme (Reed, 2001). 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 (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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