The influence of ginger as a chemopreventive agent on proliferation and apoptosis in chemically induced oral carcinogenesis

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Abstract: Cancer chemoprevention is the use of natural, synthetic, or biologic chemical agents which suppress or prevent carcinogenic progression. One of the natural ingredients that plays a role in cancer chemoprevention is ginger (Zingiber officinale Roscoe, Zingiberaceae) .It has an anti-carcinogenic activities. **Objective** :The purpose of this study is to detect the chemopreventive effect of ginger on tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4-NQ) with correlation to its anti-proliferative activity and induction of apoptosis. **Materials and Methods:** Twenty male albino rats were divided into two groups. 4-NQ was delivered to the two groups in the drinking water .The second group was given in addition, ginger tablets after grinding into fine powder added to the laboratory chow Tongue samples were obtained after thirty two weeks. Immunohistochemical staining for Caspase-3 was performed for detection of apoptosis and silver nitrate staining of nucleolar organizer regions (AgNORs) was done for estimating the proliferation of cells. The data were analyzed using Student's independent t-test and one-way analysis of variance (ANOVA). **Results:** The first group (4-NQ treated group) revealed pathological evidence of carcinogenesis .However,the second group(the ginger treated group) revealed no invasion or carcinomas.Only hyperplasia with hyperkeratosis and dysplastic lesions were observed. Apoptosis detected by caspase-3 immunostaining was statistically highly significant in the ginger treated group ($p<0.05$) meanwhile proliferation estimated by AgNOR nuclear count was statistically highly significant in the 4-NQ treated group ($p<0.05$). **Conclusion:** Ginger may have a chemopreventive effect on oral carcinogenesis through induction of apoptosis and suppression of tumour growth and proliferation. [Nature and Science 2010;8(11):44-51]. (ISSN: 1545-0740).

Key words: Carcinogenesis, cancer chemopreventive agents,ginger

1-Introduction:

Cancer chemoprevention was first defined in 1976 by Sporn, through the use of natural, synthetic, or biologic chemical agents which reverse, suppress, or prevent carcinogenic progression Sporn (1976). The success of several clinical trials in cancer prevention in high-risk populations suggests that chemoprevention is a rational and appealing strategy. In addition, Cancer chemoprevention is very attractive and has earned serious consideration as a potential means of controlling cancer incidence ,National Cancer Institute(2002)

Oral cancer is one of ten most frequent cancers in the world. Squamous cell carcinoma is the most common malignant tumour of the oral cavity.Accounting for over 90% of the malignant neoplasms in this region and is thought to arise from a progressive dysplasia of the overlying oral squamous epithelium Nishimura et al.,(2004).

Natural dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public owing to their demonstrated ability to suppress cancers Aggarwal, and Shishodia,(2006). One of these natural ingredients that plays a role in cancer chemoprevention is ginger (Zingiber officinale Roscoe, Zingiberaceae) which has been cultivated for thousands of years as a spice and for medicinal purposes Park and Pizzuto,(2002). Ginger contains some phenolic substances which generally possess strong anti-inflammatory and anti-oxidative properties and exert substantial anti-carcinogenic and anti-mutagenic activities ,Surh,(1999 and 2002). The consumed portion of the ginger plant is the rhizome that is often called “ginger root” although it is not actually a root; it is the horizontal stem of the plant that sends out the roots , White (2007). Ginger can be consumed as a fresh or dried root and is often prepared in teas, soft drinks, and breads. No specific dosing studies have been performed; however, most clinical research has used between 250 mg and 1 g of the powdered root in capsular form, taken one to four times daily Ernst and Pittler (2000) and Borrelli et al., (2005).

Ginger's root contains 6-paradol and a large amount of 6-gingerol; which is a pungent ingredient that has been found to exert various pharmacological effects such as anti-inflammatory, analgesic, antipyretic and antioxidant activity, Mustafa et al., (1993) and Aeschbach et al., (1994). It was found that these compounds suppress proliferation of human cancer cells through the induction of apoptosis Lee and Surh (1998; Lee et al., 1998 and Bode et al., (2001).

Several reports have been conducted to investigate the chemopreventive effect of ginger on the tumour growth and suppression of different human cancer cells including skin cancer Lee et al., (1998) and Park et al., (1998) gastrointestinal cancer Yoshimi et al., (1992) colon cancer ,Manju and Nalini(2005)and Dias (2006) breast cancer Nagasawa,(2002).

Yagihashi et al. (2008) found that 6-gingerol inhibited the proliferation of hepatoma cells in culture and suggested that this may be due to cell cycle arrest and apoptosis induction. In addition, it was found that 6- gingerol induces apoptosis in human cancer cells by increasing the p53 , Bax levels and decreasing the expression of Bcl-2 and Survivin genes, which resulted in the release of Cytochrome c, Caspases activation and increase in apoptotic protease-activating factor-1 (Apaf-1) Nigam et al., (2009). Meanwhile, 6-Paradol and other structurally related derivatives were found to induce apoptosis in an oral squamous carcinoma cell line through a caspase-3-dependent mechanism. Keum et al.,(2002)

Although the chemopreventive activities of ginger have been examined, very little information is available in the literature with regards to its chemopreventive effects on oral carcinogenesis. The purpose of this study is to detect the chemopreventive effect of ginger on tongue carcinogenesis induced by 4-nitroquinoline-1-oxide (4-NQ), with correlation to its antiproliferative activity and induction of apoptosis through the assessement of AgNORs and caspase-3 immunoexpression.

2-Materials and methods:

2.1.Materials

Animals

Twenty adult male albino rats weighing (150-200 gm) were selected and divided into two groups (ten animals each).They were allowed access to normal laboratory chow and drinking water. The animals were housed and caged separately in plastic cages in an air conditioned room at 22 ± 2 ˚C and 55 ± 10% humidity. The experimental procedure was conducted in compliance with ethical principles for animals' research as reviewed and approved by institutional guidelines of Kasr-Elainy animal and experimental laboratory (Faculty of Medicine, Cairo University).

2.2.Methods:

2.2.1.Induction of cancer

Carcinogenesis was induced in the animals of both group **I** and group **II** in which the carcinogen 4-NQ was obtained as a powder (Sigma, St. Louis, MO, A, cat. # N8141) and dissolved in the drinking water for rats of both groups to a final concentration of 0.02 g/l (20ppm). The prepared drinking water was changed once a week and the rats were allowed access to the drinking water at all times during the experiment

2.2.2.Ginger treatment:

Ginger was given to group **II** only from the beginning of the experiment after grinding one treated ginger tablet, 400 mg (Arab Co. pharmaceutical and medicinal plants, MEPACO, Egypt) into fine powder which was added to the laboratory chow of the rats once per day. At the 20th week of the experiment, two rats from each group were sacrificed and analyzed for precancerous and cancerous lesions. The study period was extended for thirty two weeks. After that all the remaining rats were sacrificed and the tongues were dissected.

2.2.3.Histological examination:

Tongue specimens were fixed with 10% formalin, treated with alcohol and then embedded in paraffin wax. Sections of 4µm thickness were made for routine histopathological examination with haematoxylin and eosin .Examined under light microscope.

2.2.4.Immunohistochemical analysis:

For immunohistochemical staining, paraffin embedded tissuesb were sectioned at 4 µm and collected at serial sections on positive charged slides (SuperFrost Plus-Menzel GmbH) were deparaffinized and dehydrated. Antigen retrieval was performed by boiling the slides in 10Mm citrate buffer, pH 6.0 for 20 minutes in a domestic microwave. Slides were left to cool for 30 minutes at room temperature. Sections were incubated in 3% hydrogen peroxide for 20 minutes. Novocastra protein block (RE7102 Novocastra, UK) was applied for 10 minutes after which the slides were incubated with the primary rabbit monoclonal antibody; anti-caspase 3 [(CPP32) Ab-4 Thermo Fisher Scientific, USA] diluted 1:100; for 30 min. at room temperature in a humidified chamber. After rinsing twice with TBS (Tris Buffered Saline, Amresco-USA), sections were treated with biotinylated secondary antibody (RE7103 Novocastra, UK) then labeled with streptavidin-biotin kit (RE110-k Novocastra, UK). The sections were then incubated in 3,3'diaminobenzidine (RE7190-k Novocastra, UK) for 5 minutes and counterstained with Mayer's hematoxylin (RE7107 Novocastra, UK).

2.2.5.Silver Nucleolar Organizer Regions (AgNOR) staining technique:

Equal proportions of 50% silver nitrate soln. and gelatin soln. were mixed immediately before use.

Sections were dewaxed in xylene and hydrated through ethanols to water then the slides were rinsed in distilled water. The Sections were then incubated in freshly prepared AgNOR working solution for 45 minutes at room temperature. After that the slides were washed in distilled water for one minute, then dehydrated, cleared and mounted in non aqueous mounting medium.

2.2.6.Caspase-3 and AgNOR staining assessment:

The histological sections were examined using light microscope to assess the prevalence of positive ones. For caspase-3 positive cytoplasmic immunoexpression.The percentage of positive cells was measured in the form of an area and area percent inside a standard measuring frame of area 11434.9 micrometer^2 per 10 fields by a magnification (x200) using image analysis software (Leica -Qwin) system. In addition, the number and area percent of AgNOR positive dots were counted per 10 fields using the image analysis. The nuclei that were overlapped or those with indiscernible AgNORs were excluded.

2.2.7.Statistical analysis:

Quantitative data of the image analyzer were statistically evaluated and presented as means and standard deviation (SD) values. Student's t-test was used to compare mean values of caspase-3 immunoexpression related parameters in both groups. In addition, ANOVA (analysis of variance) test was used to compare the mean values of AgNOR obtained data between the two experimental groups. The significance level was set at P 0.05.

3-Results:

At the 20th week of the experiment, examination of the tongue´s sections of from two rats of the 4-NQ treated group (group I) showed evidence of hyperkeratosis, a canthosis and signs of dysplasia in the tongue epithelium (fig.1A &B). The palate, buccal, and floor of mouth regions were not examined in detail for hyperplasia or dysplasia. At the end of the experiment; at the 32nd week, all the tongues of the mice of group I revealed pathological evidence of carcinogenesis in the form of dysplastic epithelium and invasive squamous cell carcinomas identified by the invasion of neoplastic epithelial cells into the subepithelial tissues (fig. 1C)

However, the sections of the tongues from group II (treated with ginger) revealed only hyperplastic lesions and hyperkeratosis of the epithelium at the 20th week of the experiment (fig.2A &B). Meanwhile, at the 32nd week, the tongues from the mice of the ginger treated group revealed no invasion or carcinomas, only hyperplasia with hyperkeratosis and dysplastic lesions were observed (fig.2C).

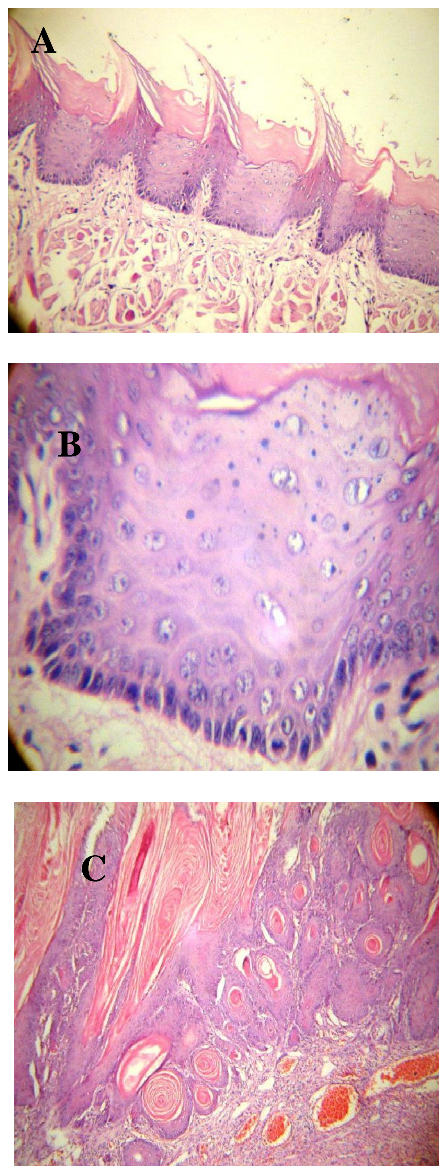
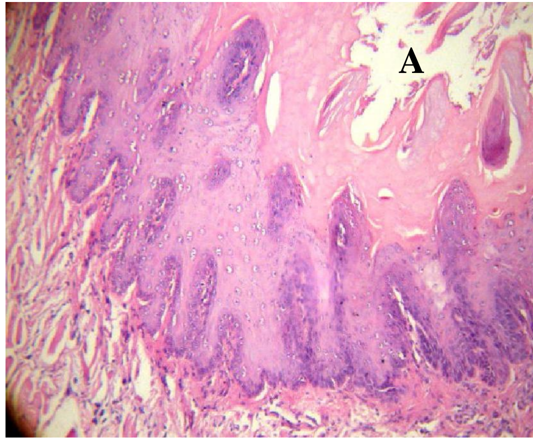
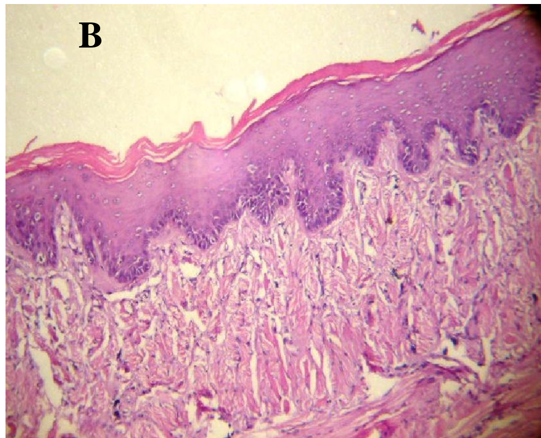


Fig.1: (A) a photomicrograph of the mouse tongue after 4-NQ treatment at the 20th week showing hyperkeratosis, acanthosis and clubbing of the rete pegs (H&E, x100). (B) A higher magnification showing hyperchromatism of the basal nuclei (H&E, x 400). (C) After 32weeks, invasive squamous cell carcinoma, cell nests and keratin pearls (arrows) (H&E, x100)





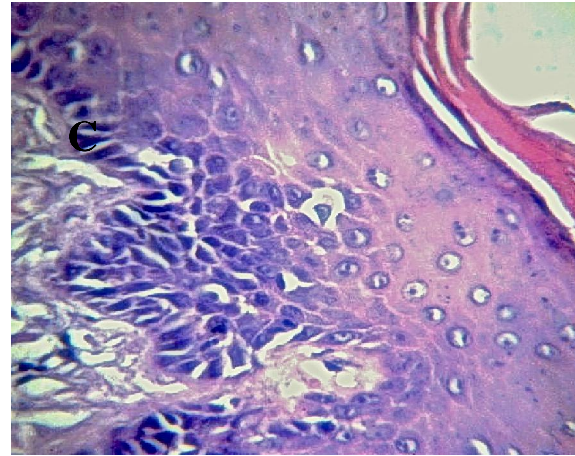
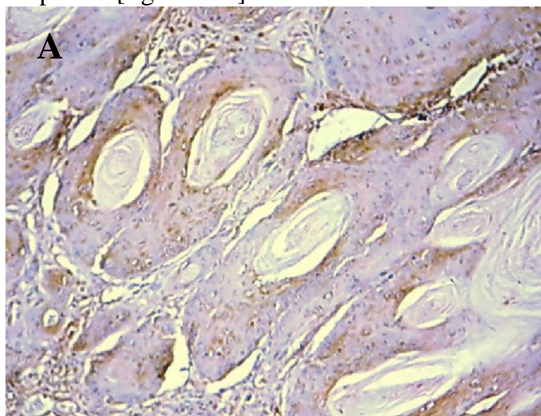


Fig.2: Group II, (A) a photomicrograph of the tongue mouse at the 20th week showing hyperkeratosis and acanthosis of the squamous epithelium (H&E, x100). (B) Inferior surface of the tongue with hyperkeratosis, acanthosis and mild dysplasia (H&E, x100). (C) At 32 weeks, basilar hyperplasia, nuclear hyperchromatism and loss of cellular adhesion (H&E, x400)

Caspase immunostaining:

Occasional immunoreactivity was observed for caspase-3 in group I (4- NQ treated group) [fig.3A &B]. On the other hand, sections of the tongues of group II revealed a higher immunoexpression of caspase-3 [fig.4A &B]



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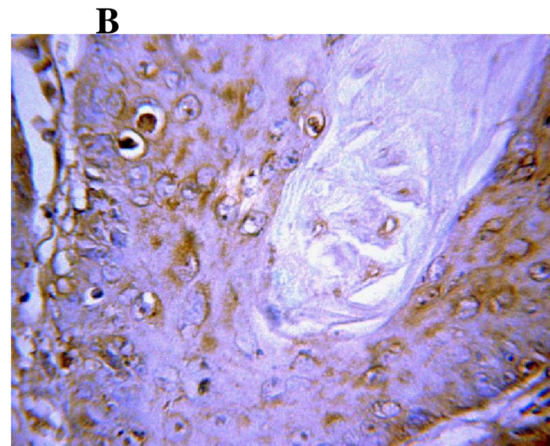

Fig.3: Group I (A) low caspase-3 immunoexpression in cell nests (original magnification, x 200). (B) A higher magnification of a cell nest showing cytoplasmic caspase-3 immunoexpression in few cells (original magnification, x 400).

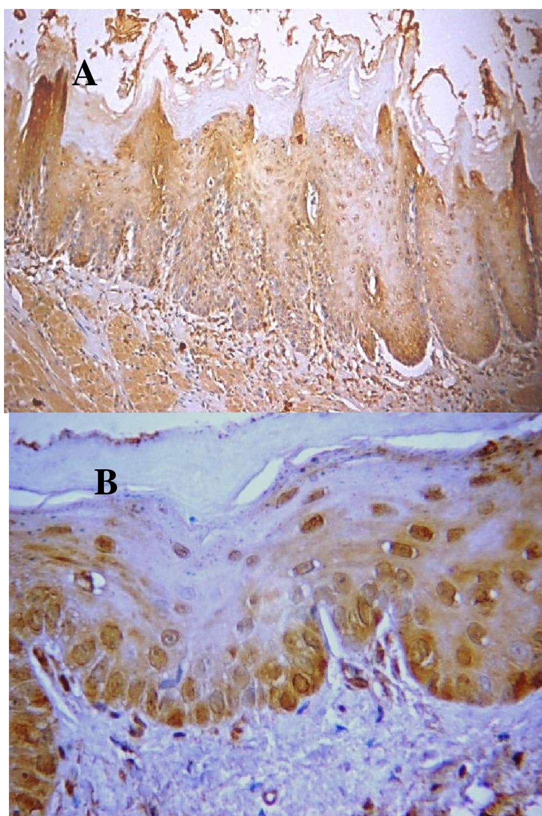

Fig.4: Group II (A) High caspase-3 immunoexpression in mild dysplasia (original magnification, x100). (B) Both nuclear and cytoplasmic immunoexpression of caspase-3 in atrophied tongue epithelium (original magnification, x 200).

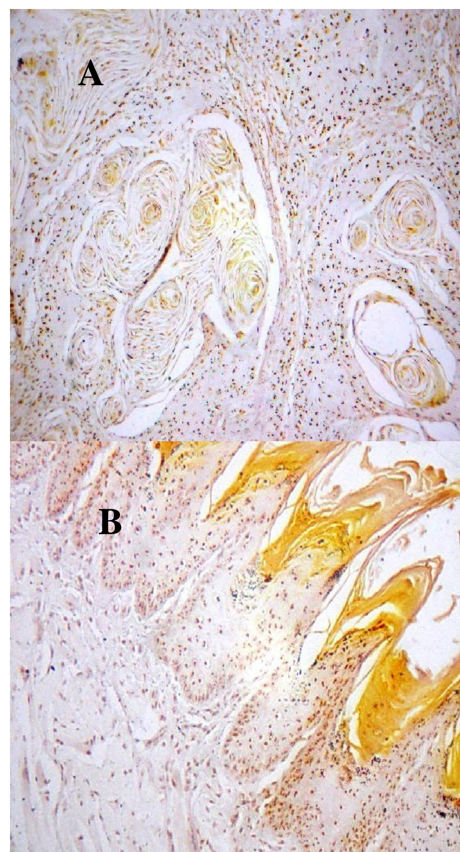

Fig.5: (A) Silver stained section of group I showing numerous silver stained nuclei in cell nests (original magnification, x100). (B) A tongue section of group II stained with silver showing less number of AgNOR dots in mild dysplasia (original magnification, x100)

AgNOR staining:

AgNOR were strictly located within the nucleus and were distinctly stained in black, being visible as dots. Silver stained dots were great in group I sections, almost all of the nuclei were stained (fig.5a), meanwhile group II revealed lesser silver stained nuclei, restricted to the basal and parabasal layers of the epithelium (fig.5b)

Results of the student's t-test revealed a highly significant area percentage of the immunoreaction of caspase-3 ($p<0.01$) between the two experimental groups (table 1& fig. 6).

Table (1): Comparing the area percentage of caspase-3 positive cells in group I and group II

Statistical profile	Group I	Group II
Mean±SD	6.28±2.63	22±7.27
Student's t-test	6.41	
P-value	<0.001*	

** Significant at P 0.05*

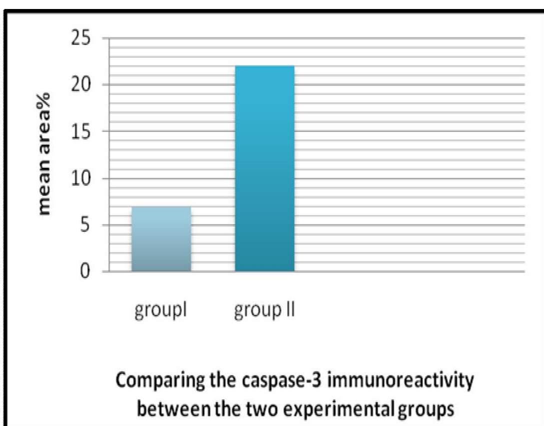

Fig.6: Bar chart illustrating the difference in area percentage of caspase-3 immunoreactivity in the two experimental groups

ANOVA test showed a highly significant area percentage of the positive AgNOR dots ($p<0.01$) between the two investigated groups (table 2& fig.7)

Table (2): Comparing the area percentage of the AgNOR positive nuclei in group I and groupII

Statistical Profile	Group I	Group II
Mean±SD	9.15±4.004	5.3±2.667
F- value	8.9352	
P- value	<0.001*	

** Significant at P 0.05*

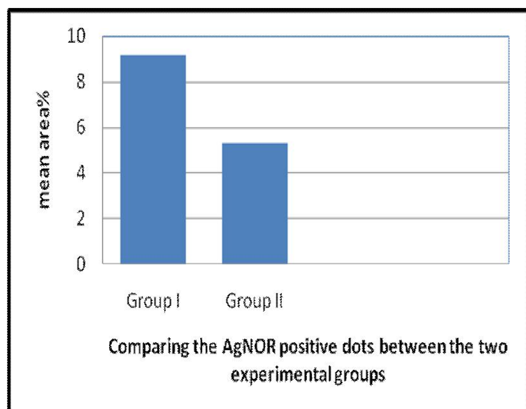

Fig.7: Bar chart illustrating the difference in the area percentage of the positive AgNOR dots in the two experimental groups

4-Discussion:

In the present study, the chemopreventive effect of ginger on oral carcinogenesis induced by 4-NQ was investigated with correlation to its anti-proliferative activity and induction of apoptosis. 4-NQ is a water soluble carcinogen that produces all the stages of oral carcinogenesis and several lines of evidences suggested that similar histological as well as molecular changes were observed in the human system Kanojia and Vaidya(2006).4-NQ was delivered in the present work in the drinking water of rats. At the end of the experiment (after 32 weeks), most of the tongue sections of group I revealed pathological evidences of carcinogenesis. This in accordance with Tang et al. (2004) who found that 4-NQ in the drinking water caused premalignant and malignant lesions in the oral cavities, observed in the mice tongues.. In addition, they found that the incidence of carcinogenesis was higher when 4-NQ was delivered in the drinking water than that when painted on the tongues.

Many dietary factors have been used as anti-cancer agents because they have low toxicity and show very few adverse side effects, Surh (2003). Ginger is one of these natural food components, which has been shown to have anti-cancer and antioxidant effects Shukla and Singh (2007). In the present study, ginger was able to reduce the incidence of oral carcinogenesis in rats of group II, no invasion or carcinomas were observed in tongue sections only hyperplasia with hyperkeratosis and dysplastic lesions. This is inconsistent with several studies done on different chemically induced rat carcinogenesis, who demonstrated low incidence of cancer formation after treatment with ginger Manju andNalini(2005);Ihlaseh et al., (2006)and Habib ety al., (2008).This suggested that ginger may play a role in the chemoprevention of oral cancer if given as a dietary component.

In addition, to my knowledge, this is the first study investigating the anti-cancer effect exhibited by ginger on oral cancer cells through its effect on proliferation and apoptosis induction. Caspases are fundemental components of the apoptotic machinery. Caspase-3 is an enzyme that becomes activated during apoptosis in a wide variety of tissues Woo (1998). In this study, the immunoexpression of caspase-3 was found to be statistically highly significant in group II (ginger treated group) which is in accordance with Lee and Surh (1998) and Lee et al. (1998) who suggested that the active compounds of ginger suppress proliferation of human cancer cells through the induction of apoptosis. Furthermore, Nigam el al. (2009) found that gingerol possesses an apoptotic potential through the release of Cytochrome c, Caspases activation and the increase in apoptotic protease-activating factor-1 (Apaf-1). In addition, Keum el al. (2002) found that paradol induced apoptosis in an oral squamous carcinoma cell line, through a caspase-3-dependent mechanism.

Nucleolar organizer regions (NORs) are the sites of ribosomal RNA which reflects protein

synthesis. AgNOR dots were the visualized structures of NORs that could be selectively stained by a silver colloid technique and could be visualized as black dots under the transmission microscope Uno et al., (1998). The results of this work showed a highly significant difference in the number of AgNOR dots between the two investigated groups where group I (4-NQ group) showed a higher number of AgNOR dots than group II. Hall et al. (1988) and Chatterjee et al. (1997) reported that the number of AgNORs rose with increasing the proliferative activity of cells, thus the number of AgNOR dots in malignant lesions is higher when compared to normal or benign lesions. Moreover, Eslami et al. (2006) concluded that there is an increase in the number of AgNOR dots with the advancement of malignancy.

Consequently, the findings of the present work suggested that ginger might exert a chemopreventive effect on oral carcinogenesis through suppression of tumour growth ,proliferation of cells and induction of apoptosis

In conclusion, the benefits provided by ginger must be viewed as part of the entire diet. In addition, further studies are recommended on determining the anticancer activity of ginger and its active components against oral cancers.

5-References:

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