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Histological and immunohistochemical evaluation of the chemopreventive role of lycopene in tongue carcinogenesis induced by 4-nitroquinoline-1-oxide

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ABSTRACT

Objective: Increasing attention has been given to the potential protective roles of specific antioxidant nutrients found in fruits and vegetables. However, there are relatively few reports on the cancer chemopreventive effects of lycopene or tomato carotenoids in animal models. Therefore, the chemopreventive efficacy of lycopene with regard to oral carcinogenesis was investigated using 4-nitroquinoline-1-oxide (4-NQO) induced tongue squamous cell carcinoma.

Materials and methods: Twenty albino rats were equally divided into 2 groups. 4-NQO was dissolved in the drinking water (20 ppm) for rats of both groups. Rats in group 2 were administered lycopene at a dose of 2.5 mg/kg body weight by intragastric intubation once a day. Incidence of oral neoplasms and histopathological changes were microscopically evaluated after 32 weeks of administration of the specific treatments. Moreover, immunohistochemical expressions of proliferating cell nuclear antigen (PCNA), E-cadherin and β-catenin were analysed in tongue specimens using an image analyser computer system.

Results: Lycopene treatment significantly decreased the incidence of 4-NQO induced tongue carcinogenesis. A decreased percentage of PCNA-positive nuclei was associated with lycopene treatment. Proliferating cells were mainly confined to the basal and parabasal epithelial cell layers. Increased E-cadherin and β-catenin immunoexpression was recorded in the lycopene treated group in comparison to the carcinogen group.

Conclusion: Results of the present study indicate that lycopene can exert protective effects against 4-NQO induced tongue carcinogenesis through reduction in cell proliferation and enhanced cellular adhesion, suggesting a new mechanism for the anti-invasive effect of lycopene. Further studies are needed to provide more definitive answers to the question of the anticancer effects of lycopene.

1. Introduction

Every year more than 300,000 new cases are being diagnosed with oral squamous cell carcinoma (SCC) world wide. Oral cavity cancer and cancer of oropharynx represents the sixth most common malignancy globally.[1,2] Typically, oral cancer development is a slow and cumulative process, which occurs after exposure of the entire epithelial surface to the repeated insult of carcinogens. This process strongly supports the rationale for a prevention strategy that will inhibit, delay, or reverse carcinogenesis before it becomes invasive disease. Chemoprevention fulfils this strategy and offers a promising treatment for oral cancer.[3]

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Epidemiological studies have indicated that populations that consume food rich in fruits and vegetables have a lower incidence of cancers.[4,5] A meta-analysis showed that a high vegetable intake decreases the risk for oral cancers by almost half.[6]

High consumption of tomatoes effectively decreases the risk for cardiovascular disease and cancer by improving the antioxidant capacity.[7] Tomatoes are rich sources of lycopene, a natural pigment synthesised by plants and microorganisms.[8] It is an acyclic isomer of β-carotene with no vitamin A activity.[9] Because of its antioxidant properties, lycopene has gained attention and became one of the most studied chemopreventive agents. It is believed that the mechanisms underlying the inhibitory effects of lycopene on carcinogenesis and mutagenesis could also involve improving cell to cell communication,[10] interference with cell proliferation,[11–13] induction of gap-junctional communication,[14] inhibition of cell cycle progression and modulation of signal transduction pathways,[7] in addition to induction of apoptosis.[15]

Lycopene has been postulated to be the protective compound against prostate cancer.[16,17] In cell culture, lycopene strongly inhibited proliferation of human endometrial, mammary and lung cancer cells.[11,18] Lycopene treatment also resulted in a concentration-dependent reduction in HL-60 leukaemic cells' growth, which was accompanied by inhibition of cell cycle progression in the G0-G1 phase.[19]

Concerning oral cancer, an inverse association was observed between lycopene and buccal pouch squamous cell carcinomas in hamsters.[20] Moreover, the intake of daily lycopene capsules effectively improved oral leukoplakia lesions in a group of patients.[21]

To develop new methods and improve existing protocols for diagnosis and treatment, an animal model that mimics the natural course of SCCs was mandatory. Application of 4-nitroquinoline-1-oxide (4NQO) to rat tongues is a reliable procedure for inducing oral SCCs.[22] 4NQO is a powerful carcinogen in several organs, and it can specifically induce tongue SCC when applied in low concentrations via drinking water. The resulting sequential changes and morphological features resemble those seen during the progression of human tongue squamous cell malignancy. Moreover, level of DNA damage is directly related to the severity of the histological changes.[23] Therefore, 4NQO-induced rat tongue SCC is an excellent model for studying early events in oral carcinogenesis[24,25] and to investigate synthetic and natural agents for chemopreventive potential.[26–28]

Regarding the need for information focusing on the chemopreventive potential of naturally occurring compounds, the current study was conducted to evaluate the role of lycopene treatment on 4NQO induced tongue carcinogenesis through quantitative evaluation of the proliferative cell nuclear antigen (PCNA), β-catenin and E-cadherin immunoexpression.

2. Materials and methods

A total of 20 male albino rats were used in this study. Rats were maintained in plastic cages in an air conditioned room at 22 ± 2 °C and 55 ± 10% humidity with free access to standard pellet diet and drinking water. The experimental procedure was conducted in compliance with ethical principles for animals' research as reviewed and approved by institutional guidelines of Kasr Al-ainy animal and experimental laboratory (Faculty of Medicine, Cairo University).

After 1 week of acclimatisation, rats were randomly allocated to two groups (10 rats each). 4-NQO was obtained as a powder (Sigma, St. Louis, MO, USA, cat. # N8141) and dissolved in the drinking water for rats of both groups to a final concentration of 0.02 g/l (20 ppm), and the water was changed once a week. In addition, rats in group 2 were administered lycopene (Sigma, cat. # L9879) at a dose of 2.5 mg/kg body weight by intragastric intubation once per day.

2.1. *Specimens preparation*

Animals of both groups were sacrificed after 32 weeks of administration of the specific treatments. The tongue was cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin and embedded in paraffin blocks. Each tissue was totally submitted to multiple transverse sections for histological processing.

2.2. *Histopathological analysis*

Histopathological evaluation was performed by light microscopy. Tongue sections were graded as normal, hyperplasia, carcinoma in situ, dysplasia, and carcinoma per animal, as modified from Ribeiro et al.[29]

2.3. *Immunohistochemistry*

Sections at 5 μm thickness were deparaffinised in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval, slides were placed in 0.01 M citrate-buffer pH 6.0 and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3% H_2O_2 for 20 min at room temperature. Sections were further incubated in blocking serum for 30 min. For application of the primary antibodies, sections were incubated overnight at 4 °C with anti-PCNA (proliferating cell nuclear antigen) monoclonal antibody (PC10, 1:20 diluted, DAKO M0879, Glostrup, Denmark), anti-E-cadherin antibody, 1:400 diluted (Santa Cruz Biotech., Santa Cruz, CA, USA), or β-catenin mouse monoclonal antibody, 1:100 diluted (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, sections were incubated with biotinylated secondary antibody (LSAB2, Dako, Glostrup, Denmark) for 30 min, washed in phosphate buffered saline (PBS) three times for 10 min each. LSAB2-streptavidin-peroxidase detection reagent (DAKO, Glostrup, Denmark) was subsequently added evenly over the sections and incubated for 30 min. Finally, the reaction was developed using 3,3′-diaminobenzidine tetrahydrocloride (DAB) for 5 min. Slides were briefly counterstained in haematoxylin and dehydrated, and cover slips added. As negative controls, tissue sections were processed in parallel by omitting incubation with the primary antibodies.

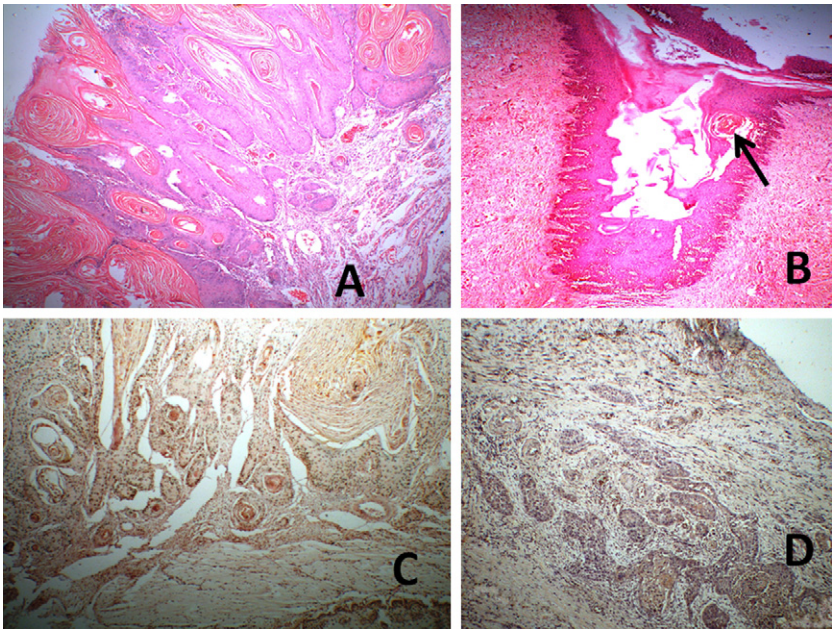

Fig. 1 – Photomicrographs of group I revealing: (A) Well differentiated squamous cell carcinomas infiltrating deep in the underlying connective tissue (H&E 40×). (B) Severe dysplasia exhibiting top-to-bottom changes with keratin pearl formation (arrow), (H&E 40×). (C) PCNA-positive nuclei in the malignant epithelial cell nests (anti PCNA 40×). (D) Weak cytoplasmic E-cadherin immunoexpression (anti E-cadherin 40×).

2.4. *Quantitative and statistical analyses*

The incidence of development of the histopathological changes (hyperplasia, carcinoma in situ, dysplasia, carcinoma) was compared using the chi-square test. For immunohistochemical evaluation, epithelial cells with nuclei showing brown staining were considered PCNA-positive cells. The PCNA-positive cells in 100 basal and parabasal cells of the tongue were counted in five high-power fields. The average percentage of PCNA-positive cells and its standard deviation value for each group were obtained.

Immunostaining was examined by the image analyser computer system with the software Leica Quin 500 (Leica Microsystems Inc., Heerbrugg, Switzerland). The E-cadherin and β-catenin immunoreactivity was measured in the form of an area and area percent in a standard measuring frame per 10 fields using the magnification 400× by light microscopy transferred to the monitor. The areas of the most intense staining were masked by a blue binary colour that could be measured by the computer system. The measured values were expressed as mean ± standard deviation. The data were analysed statistically using Student's *t*-test. A p value below 0.05 was considered significant.

3. Results

3.1. *Clinicopathologic findings*

In group I, lesions detected on the surface of the tongue on macroscopic examination were either exophytic (protruding from the tongue) or endophytic (ulcers) that varied in size. These were mainly observed in the dorsal site of the root of tongue.

In group II, no gross lesions were visible in specimens that were subsequently found to be hyperplasia or dysplasia on histological examination. An exophytic mass of ill-defined outline was observed in the case that revealed malignancy (SCC) on microscopic analysis.

3.2. *Histological findings*

In group I, the incidence of squamous cell carcinomas (SCC) induced by 4-NQO was 80%. Histologically, all 4NQO-induced tongue neoplasms were found to be well differentiated squamous cell carcinomas with evidence of keratin formation. Tumour cells having pleomorphic, hyperchromatic nuclei exhibited altered nuclear/cytoplasmic ratio. The basement membrane appeared discontinuous. The tumours spread into the submucosa and underlying muscle fibres of the tongue, forming small nests with typical keratin pearl formation (Figs. 1 and 2). Carcinoma in situ was noted in 20% of the animals of group I. These lesions exhibited loss of polarity in the epithelial cells, nuclear pleomorphism and hyperchromatism, abnormal single cell keratinisation (dyskeratosis), cytologic disorganisation and increased or abnormal mitoses without submucosal tissue invasion. Chronic inflammatory cell infiltration was noted in the connective tissue (Figs. 1 and 2).

In group II, lycopene significantly decreased the incidence of squamous cell carcinoma. Microscopic examination revealed a single case of well-differentiated SCC (10%), one case of carcinoma in situ (10%), whilst four cases (40%) exhibited epithelial dysplasia, with different degrees of atypia. Dysplastic lesions demonstrated disorientation of cells,

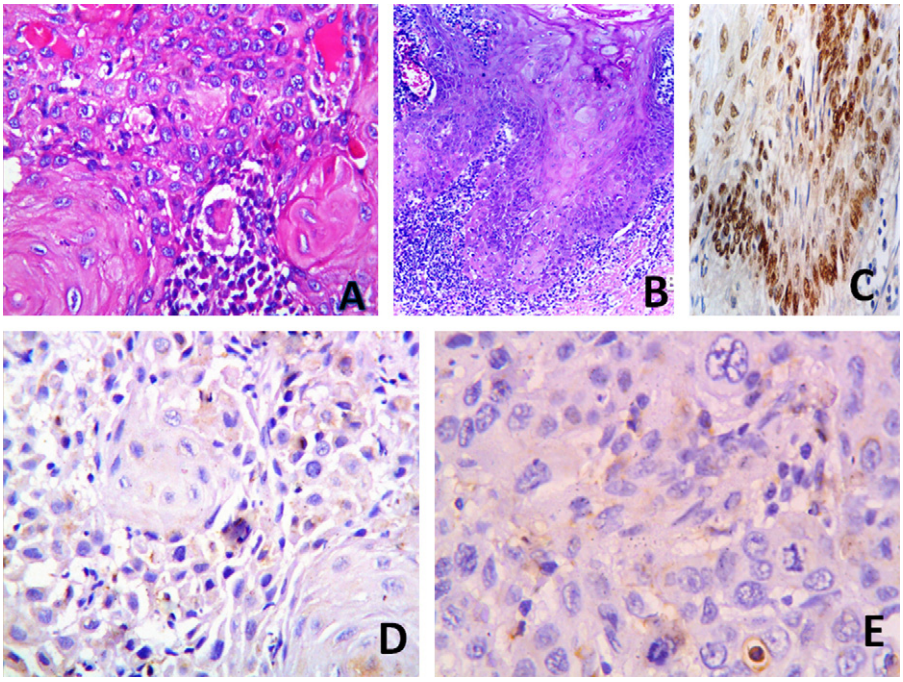

Fig. 2 – Photomicrographs of group I revealing: (A) Well differentiated squamous cell carcinomas forming small nests with typical keratin pearl formation (H&E 200×). (B) Severe dysplasia exhibiting top-to-bottom changes without submucosal tissue invasion (H&E 100×). (C) PCNA-positive nuclei in the basal and suprabasal layers of epithelium (anti PCNA 200×). (D) Weak cytoplasmic E-cadherin immunoexpression (anti E-cadherin 200×). (E) An almost absent β-catenin immunostaining in the induced squamous cell carcinomas (anti β-catenin 400×).

cellular pleomorphism, nuclear hyperchromatism, proliferation of basal cells, abnormal keratinisation and elongation of epithelial rete pegs (Figs. 3 and 4). Hyperplasia with clearly defined basement membrane was seen in 4 cases (40%). Using the Chi square to compare the incidence of development of carcinomas, carcinoma in situ, dysplasia, or hyperplasia a highly statistically significance difference (Chi.Sq = 13.778; $p = 0.00322345$) was detected between groups I and II.

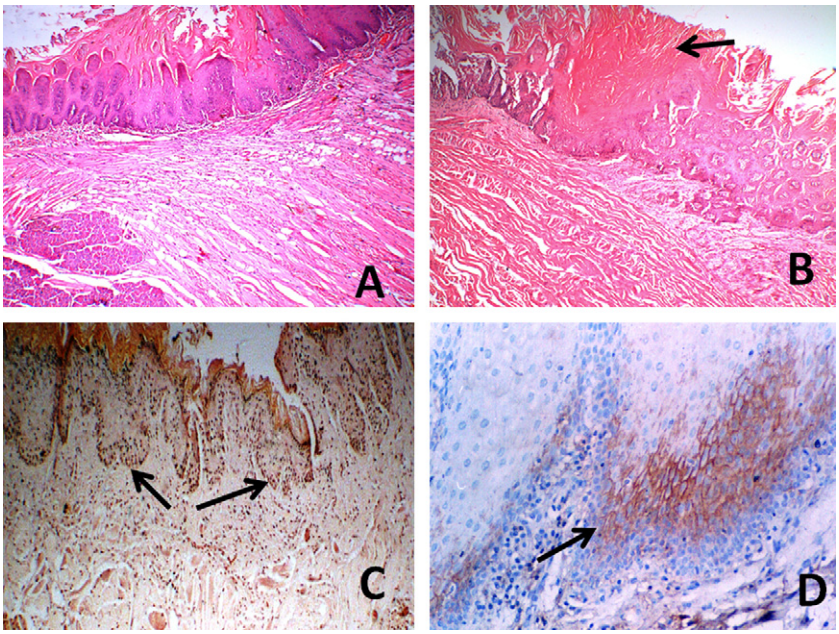

Fig. 3 – Photomicrograph of group II revealing: (A) Mild dysplastic lesion (H&E 40×). (B) Moderate dysplastic lesion with marked hyperkeratosis (arrow), (H&E 40×). (C) PCNA-positive nuclei in the basal and parabasal cells of the dysplastic lesions (arrows), (anti PCNA 40×). (D) Membranous E-cadherin immunoexpression (arrow), (anti E-cadherin 100×).

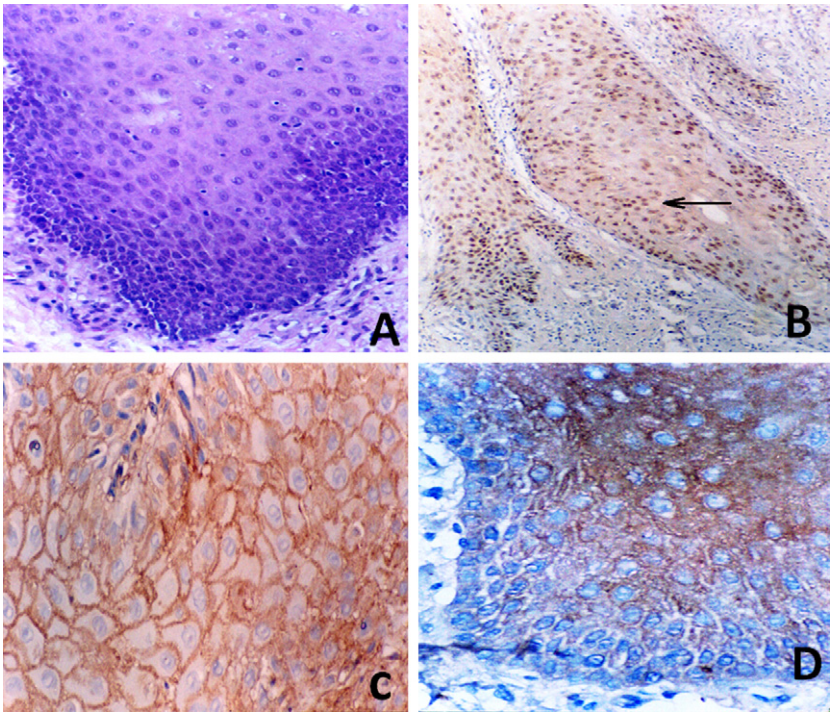

Fig. 4 – Photomicrograph of group II revealing: (A) Mild dysplasia confined to the basal third of the epithelium (H&E 100×). (B) PCNA expression in the basal and parabasal epithelial cells of the dysplastic lesions (arrow), (anti PCNA 200×). (C) Membranous E-cadherin immunoexpression (anti E-cadherin 400×). (D) β-Catenin immunostaining in almost all layers of the hyperplastic epithelium (anti β-catenin 200×).

3.3. *Immunohistochemical findings*

Whilst immunostaining of PCNA showed nuclear localisation, E-cadherin and β-catenin were found in the membrane and cytoplasmic regions. In group I (4-NQO), the PCNA-positive nuclei were present in both the basal and suprabasal layers of epithelium of the tongue and throughout the tumour tissue (Figs. 1 and 2). The mean percentage of PCNA positive cells was (41.8 ± 12.2). In lycopene treated group (group II), PCNA expression merely appeared in the basal layer of the epithelium in the hyperplastic cases. PCNA expression was more obvious in dysplastic lesions and appeared in the basal and parabasal epithelial cells (Figs. 3 and 4). In the single SCC developed in the lycopene treated group, positive PCNA expression appeared in the nuclei of the invading malignant cells. Comparing the 2 groups, The mean percentage of PCNA positive cells was significantly different ($p = 0.0223^*$).

In group I (4-NQO), E-cadherin demonstrated a weak cytoplasmic expression pattern without presence of membranous staining (Figs. 1 and 2). Immunoexpression was higher in the lycopene treated group (group II) and demonstrated a membranous pattern (Figs. 3 and 4), however, the difference between the 2 groups did not reach the level of statistical significance ($p = 0.0588$).

Cytoplasmic/cell membrane β-catenin immunostaining was noted in the dysplastic lesions in group I (4-NQO), but was almost absent in the carcinomas (Figs. 1 and 2). In the lycopene treated group (group II), β-catenin immunostaining was observed in almost all layers of the epithelium (Figs. 3 and 4). Quantitative analysis revealed a significantly increased β-

Table 1 – Histopathological changes and mean values (mean ± standard deviation) of expression of PCNA, E-cadherin and β-catenin in both studied groups.

	4-NQO (10 rats)	4-NQO + lycopene (10 rats)	*p* value (*t*-test)	*t* value (*t*-test)
Carcinoma (well-differentiated)	8 (80%)	1 (10%)		
Carcinoma in situ	2 (20%)	1 (10%)		
Dysplasia	0 (0%)	4 (40%)		
Hyperplasia	0 (0%)	4 (40%)		
Normal epithelium	0 (0%)	0 (0%)		
PCNA	41.8 ± 12.2	29.7 ± 9.25	0.0223*	2.4992
E-cadherin	6.24 ± 3.27	10.98 ± 6.67	0.0588	2.0178
β-Catenin	8.59 ± 2.18	29.78 ± 9.43	<0.0001*	6.9233

* Statistically significant.

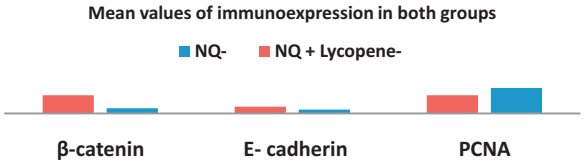

Fig. 5 – Mean values of expression of PCNA, E-cadherin and β-catenin in both studied groups.

catenin expression in the lycopene treated group ($p < 0.0001$). Histopathological changes and mean values of expression of PCNA, E-cadherin and β-catenin in both studied groups are summarised in Table 1 and Fig. 5.

4. Discussion

Functional foods are promising candidates for chemoprevention because of their safety and the fact that they are not perceived as medicine. However, there is paucity of information with respect to lycopene chemopreventive potential in oral cancer.

Animal models are useful for testing putative chemopreventive agents before embarking on clinical trials. In the present study, 4-NQO administration (20 ppm) in the drinking water of experimental rats induced tongue squamous cell carcinomas after 32 weeks of treatment. This was consistent with previous studies[23–25] and provided additional evidence justifying the use of 4-NQO in animal models of tongue carcinogenesis.

In the current study, premalignant and malignant changes following 4-NQO application were local and occurred in the setting of morphologically normal mucosa. Moreover, there was a relatively long latency period before malignancy developed, and cancer formation was preceded by a hyperplastic and dysplastic stage. This indicates that the rat model of 4NQO-induced oral carcinogenesis simulates aspects of human oral cavity carcinogenesis. The major disadvantage of the rat 4-NQO model was the long time (32 weeks) required for carcinogenesis.

The chemopreventive effect of lycopene on tongue carcinogenesis is consistent with previous reports, in which it has been shown to inhibit mammary tumour formation,[30] liver preneoplasia,[31] lung neoplasia[32] in rodent models, and 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis in hamsters.[33] Although the aforementioned studies demonstrated a carcinogenesis inhibitory effect in certain organs, some other results were conflicting.[34,35] An important issue concerning these data is that many variables differ from one study to another, including lycopene doses, forms and routes of administration, tumour induction protocol, strain-specific effects and treatment schedule.

In contrary to the findings of the present study, Müller et al.[36] reported that lycopene did not alter significantly the incidence of DMBA induced squamous cell carcinoma in hamster buccal pouch. However, these authors administered lycopene 10 weeks after DMBA application and thus concluded that lycopene may not be sufficient to suppress tumour formation in the post initiation phase. Another variable that should be considered when comparing the findings of chemoprevention studies is the tissue under investigation. This is of particular importance in the oral cavity since difference in the molecular pathways that operate in the development of carcinoma of tongue and buccal mucosa have been reported.[37]

In the current study, induced tongue carcinogenesis was associated with overexpression of PCNA and decreased expression of β-catenin and E-cadherin. PCNA is a 36 kDa nonhistone nuclear acidic protein expressed in the nuclei of proliferating cells during S-phase.[38] Moreover, PCNA is one of the helper proteins of DNA polymerase Ơ, which is a key protein in cell cycle regulation.[39] Besides proliferation, PCNA also exhibits anti-apoptotic functions. PCNA interacts with Gadd 45, a growth arrest and DNA damage protein, as well as MyD118, a myeloid differentiation primary response protein. These interactions inhibit apoptosis and differentiation and promote tumour cell growth.[40] Therefore, PCNA expression has been considered to reflect the proliferation rate of tumour cells. Shin et al.[41] found a gradual increase in PCNA expression during progression of normal epithelium from hyperplasia through dysplasia to oral squamous cell carcinoma. Thus, overexpression of PCNA observed in the present study reflects increased cell proliferation in 4-NQO induced tumours as previously reported by Kaplan et al.[24]

In the 4-NQO group, PCNA-positive cells were observed more frequently at parabasal cell layers, and the percentage of PCNA positive-cells was significantly higher compared to the lycopene group. It is well known that the basal layer of the oral epithelium is the location of the normal proliferating cell compartment, whereas suprabasal layers are only spaces of cellular maturation.[42] Hence, any sign of proliferative cellular activity beyond the basal layer indicates that these dysplastic cells are more proliferative and of higher malignant potential.

Quantitative analysis showed that PCNA expression was suppressed in the lycopene treated group. This finding is consistent with Livny et al.[10] who reported that lycopene inhibited the proliferation of the human oral cancer cell line (KB-1) in G1 phase to approximately 10% of control cell numbers. Similarly, Cheng et al.[43] reported that lycopene suppressed cell proliferation at the G0/G1 phase with a significant decrease in PCNA expression in the quid extract (BQE)-induced hamster oral cancer and in human (KB) cell models. The recorded inhibitory effect of lycopene on PCNA expression could be partially attributed to its interference in the insulin growth factor (IGF-1) receptor signalling, leading to suppressed cell cycle progression, as reported in mammary cancer cells (MCF-7).[18]

The epithelial calcium dependent adhesion molecule (E-cadherin) and β-catenin complex plays a critical role in the epithelial cell-cell contact and maintenance of intercellular adhesion.[44] Reduced cell adhesive properties of this complex have been reported to be associated with tumour development and progression in head and neck squamous cell carcinomas.[45] In the present study, decreased E-cadherin expression was noted in 4-NQO carcinogenesis. This finding accords with the reported progressive reduction in E-cadherin immunoreactivity in oral precancerous lesion followed by primary oral SCC.[46] In the induced carcinomas, E-cadherin demonstrated a weak cytoplasmic expression pattern without presence of

membranous staining. This is in consistence with the reported alteration in the expression of this molecule in oral SCC.[47,48] Differential expression with cytoplasm shifting of E-cadherin has also been demonstrated in some oral precancerous lesions, compared with the normal oral mucosa.[48] According to Fujita et al.,[49] a redistribution of the E-cadherin complex out of tight junctions can affect its functions in cell-cell adhesion and can increase its degradation by cytoplasmic endocytosis.

In the lycopene treated group, E-cadherin immunoreactivity was higher compared to group I and demonstrated the typical membranous pattern. These observations clearly indicate that lycopene is able to prevent the tumour-related alterations in the expression and localisation of E-cadherin and can consequently preserve its crucial role in cell adhesion, thus maintaining normal epithelial polarity and inhibiting invasion.

In the current 4-NQO tongue carcinogenesis model, β-catenin immunoreactivity was almost absent in the induced carcinomas. This observation accords with previous studies documenting reduced catenin expression in oral SCC.[50,51] Moreover, an inverse relationship was reported between the expression of β-catenin and the degree of cellular differentiation, together with a reduction of membrane expression of catenins in more undifferentiated cells of human oral SCC.[52] This seems to suggest that catenins physiology alterations may be involved in the transformation process.[51]

Quantitative analysis demonstrated higher levels of β-catenin cytoplasmic/cell membrane expression in the hyperplastic and dysplastic lesions of the lycopene treated group in the present study. These findings denote enhanced cellular adhesion and suggest the anti-invasive effect as a new mechanism for the chemopreventive role of lycopene, in consistence with previous reports dealing with other tissues.[53,54]

5. Conclusion

The present study clearly demonstrates that inhibition of cell proliferation and induction of cell adhesion may be major mechanisms through which lycopene exerts its oral anticarcinogenic properties. There is a rapid need for more large scale studies focusing on this naturally occurring compound to ascertain these observations and hopefully confirm their role in oral cancer chemoprevention.

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Competing interests: None declared.

Ethical approval: The research was approved by the review board of the animal and experimental laboratory (Faculty of Medicine, Cairo University, Egypt).

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