



## The chemopreventive role of aged garlic extract against chemically induced tongue carcinogenesis

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**Abstract:** Tongue tumors were induced in rats using Diethylnitrosamine (DEN). Aged Garlic extract (AGE) exhibits anticarcinogenic effects. This study aimed to investigate the protective effect (AGE) against DEN induced tongue cancer in rats. Twenty-four Wister rats were divided into 4 groups. In lateral surface of the tongue; Control Groups I and II, received normal saline, while Groups III and IV were injected with 60 mg/kg DENA in a dose of 0.25ml (15mg/rat of 200g). Groups II and IV received pre-treatment with aged garlic extract 250 mg/kg orally daily for 2 weeks. Specimens for clinical and histopathological examinations were retrieved after 3 and 6 weeks. Although clinical and dysplastic histopathological changes were observed in Group 3 and 4, yet, less aggressive features of malignancy were associated with AGE. Better designed study with different doses "DEN and larger samples of animals, are needed to expand the understanding of the chemopreventive effect of AGE as dietary factor to cancer.

**Key words:** Aged garlic extract; tongue; Diethylnitrosamine; induced carcinogenesis; Oral cancer; cancer prevention.

### Introduction

Oral cancer is one of the most common human neoplasms, being the sixth most common malignancy worldwide (1), (2). While its incidence varies greatly in different countries, it is greater in developing countries (3), (4), with an increased incidence noted among young adults particularly males, which is usually associated with exogenous causative factors such as smoking or chewing tobacco (5-8). A scientific understanding of the molecular mechanism of cancer development in the absence of these factors can help in its prevention, improvement and introduction of new forms of treatment (9).

Nitrosamines can cause cancers in a wide variety of animal species, a feature that suggests that they may also be carcinogenic in humans. At present, available epidemiological evidence from case-control studies on nitrite and nitrosamine intake supports a positive association with gastric and esophageal cancer risk (10). Diethylnitrosamine (DEN) is formed by the interaction of nitrite with diethylamine, and by the action of nitrate reducing bacteria. Oral and pharyngeal tumors and/or osteochondroma in the root of the tongue have developed in rats treated with DEN (11-14).

However, there is limited information in the literature concerning the carcinogenic effect of DEN on the oral cavity.

Garlic (*Allium*) is used as a vegetable spice and medicinal herb. Garlic exhibits a widely range of properties including immunomodulatory, hepatoprotective, antimutagenic, anticarcinogenic and an antioxidant effects (15,16). Aged garlic extract (AGE) has been produced by a number of prolonged extraction processes from fresh garlic (17-18). It is an odorless compound, characterized by having antioxidant and free radical scavenging properties and an anticarcinogenic effect (19, 20). Therefore, the present study was directed to investigate the possible protective effect of aged garlic extract against DEN, potentially induced tongue cancer in rats.

### Materials and Methods

#### Animals and drugs

A total of 24 Wister albino rats, 8-10 weeks of age, weighing 180-200 g were included in our study. These animals were of our institute's own outbreeding stock (King Fahd Medical Research Center, King Abdulaziz University). The animals were housed in a conditioned atmosphere and kept on a standard diet and water *ad libitum* in accordance with the institution's ethical standards

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of the responsible committee on animal experimentation.

Aged garlic extract (Kyoli) was kindly provided by Wakunaga of America (Mission Viejo, CA). It is prepared by soaking sliced raw garlic in 15-20% aqueous ethanol for at least 10 months at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The content of water-soluble compounds is relatively high, while that of oil-soluble compounds is low. The AGE used in this experiment contained 28.6% extract solids (286mg/ml), and S-allyl cysteine, the most abundant water-soluble compound in AGE was present at 1.47mg/ml/ Diethylnitrosamine (DENA, Sigma chemical Co, USA). DENA was dissolved in 0.9% sterilized saline and injected submucosally on the lateral surface of the tongue in a dose of 0.25ml (15mg/rat of 200g.weight).

### Experimental design

**Twenty-four male Wister rats were divided into 4 main groups as follows:** Two groups (1 and 2) were used as a control and consisted of 4 animals each. Both groups received normal saline in the lateral surface of tongue and distilled water P.O. Group (2) in addition, received aged garlic extract 250 mg/kg orally daily for 2 weeks. Experimental Groups (3) and (4); eight animals each, were injected with 60 mg/kg DENA in the lateral surface of the tongue. Group (4) animals received pre-treatment with garlic for 2 weeks. At week 3 and 6 after DENA administration animals were sacrificed and their tongues were clinically examined and specimens from tongue and site of injection were fixed on 10% formaline for histopathological examination. Specimens for the control groups were retrieved at the same corresponding intervals.

## Results

### Clinical results

Animals in both control groups (1 and 2) maintained stable weight throughout the time of the study. Clinically the animals of the control group had no signs of ulcerations or white or red patches at the injection sites. For Group 3 treated with DENA at the 3<sup>rd</sup> post injection week, all the animals appeared with varying degrees of weight loss as depicted in table 1.

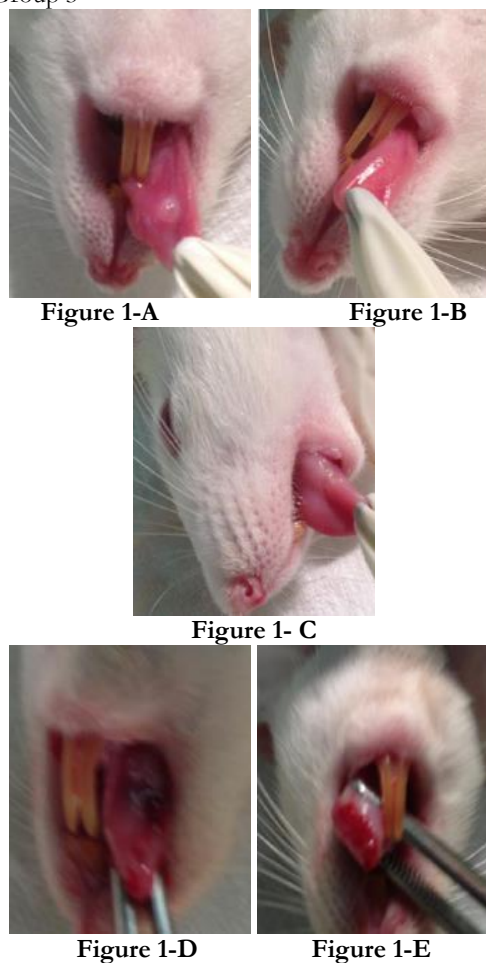
**Table 1:** Weight changes in study group animals

Date	Group I	Group II	Group III	Group IV
Baseline	192 g	194,3 g	193,7 g	190 g
3 <sup>rd</sup> week	193 g	200 g	181,2 g	208, 4 g
6 <sup>th</sup> week	198 g	204 g	165,9 g	187, 7 g
Mean	193 g	199.4 g	180,2 g	197,03 g

Clinical observations showed nodular whitish lesions in 2 animals (Figure 1-A), reddish discoloration and loss of papilla on the tip and

base of the tongue in 3 animals (Figure 1-B) and ulceration on the lateral surface of the tongue in the remaining 3 animals (Figure 1-C). At the 6<sup>rd</sup> post-injection week, one animal died. The remaining animals showed remarkable weight loss table -1. Most clinical changes varied from smooth shiny white and red lesions in 2 animals, to dark ulceration with indurated base in 3 animals (Figure 1-D). Two of the animals showed a bleeding indurated tongue ulcer extending to the floor of the mouth (Figure 1-E).

**Figure 1:** Photograph of clinical tongue lesions of Group 3



**Figure 1-A:** Nodular whitish lesion

**Figure 1-B:** Reddish discoloration and loss of tongue papilla

**Figure 1-C:** Ulceration on the lateral surface of the tongue

**Figure 1-D:** Dark ulceration with indurated base

**Figure 1-E:** Bleeding indurated tongue ulcer extending to the floor of the mouth

For Group 4 who received 2 weeks pre-treatment with AGE, animals appeared overweight and hyperactive at the 3<sup>rd</sup> post injection week (Table 1). The clinical changes were generally less severe when compared to group 3. However, changes varied from smoothing of the tongue mucosa to bluish and red discoloration in 5 animals, while, 2 animals showed white nodular patches with teeth over-eruption (Figure 2-A), and only one showed superficial ulcerations (Figure 2-B). At the 6<sup>th</sup>

post-injection week, the animals appeared less active and with loss of weight, yet with higher average weights when compared with group 3 (Table 1). The clinical changes appeared progressive but less severe than group 3. 5 animals showed varying degrees of tongue enlargement with white and/or red patches. Two animals showed superficial ulceration, while three animals suffered from indurated ulcers (Figure 2-C).

**Figure 2:** Photograph of clinical changes in Group 4



**Figure 2-A**

**Figure 2-B**



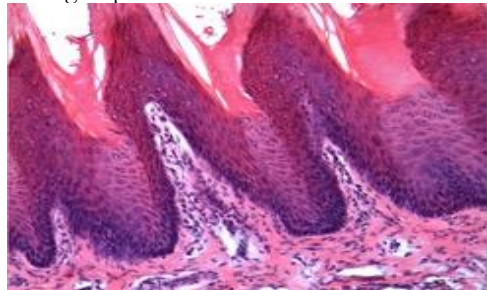
**Figure 2-C**

**Figure 2 A:** White nodular patches with teeth over-eruption

**Figure 2 B:** Superficial Ulceration

**Figure 2 C:** Indurated ulceration

**Figure 3:** Photomicrograph of a specimen of the control group



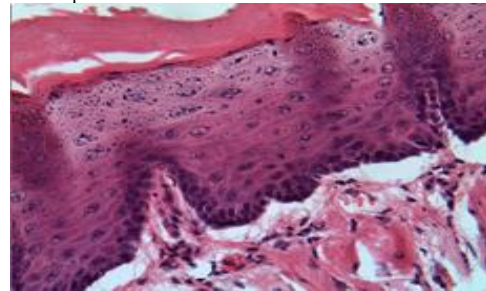
\*Normal keratinized stratified squamous epithelial cell layers with well-defined basal cell layer.

### Histopathological results

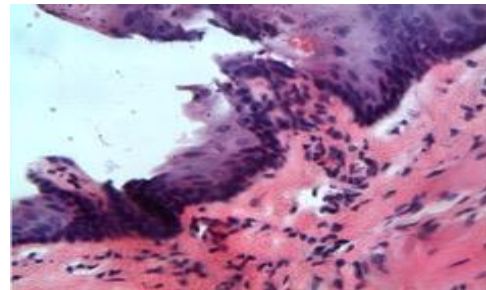
Upon histological examination, group 1 showed normal keratinized stratified squamous epithelium with inflammatory infiltrate of the underlying connective tissues with no signs of dysplasia or malignant changes was noted (Figure 3). On photomicrograph views, specimens from the 3rd post injection weeks depicted varying degrees of dysplasia and premalignant changes and all animal developed features of invasive carcinoma by the 6th post injection week including animals of

Group 3 (Figure 4-A & B) & Group 4 (Figure 5-A & B).

**Figure 4:** Photomicrograph of tongue specimens of Group 3

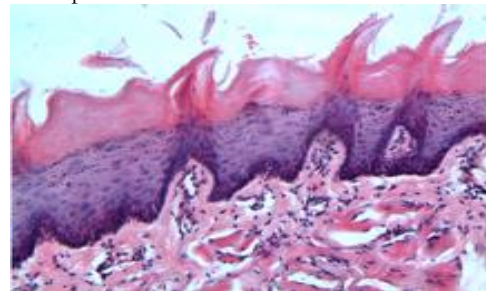


**Figure 4-A:** At 3 weeks post injection hyperplasia of basal layer. Karyorrhexis with hyperkeratosis, epithelium acanthosis, polymorphic differentiation of prickle cell layer and disorganization of Connective tissue.



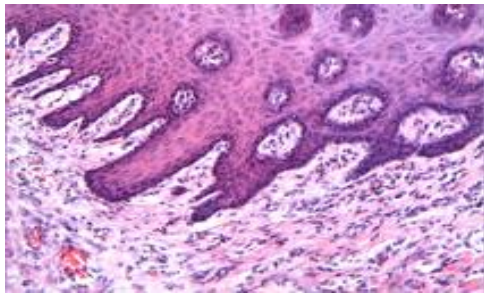
**Figure 4-B:** At 6 weeks post injection wide ulceration of surface epithelium and faintly stained prickle cell cytoplasm. The basal cells show pleomorphism with deep basophilic characters and mitosis, epithelial migration in the lamina propria, invading the epithelial layer with hyalinization and deeply stained cells.

**Figure 5:** Photomicrograph of tongue specimens of Group 4



**Figure 5-A:** At 3 weeks post injection showing invasion and disorganization of the epithelium thinning of the basement membrane with cellular hyperpigmentation and disorganization of the lamina propria with micro invasion of CT beyond the basement membrane and hyper vascularization.





**Figure 5-B:** At 6 weeks post injection showing ulceration and dysplastic changes of the epithelium. Disorientation of the cells with increased vascularity within the epithelium and microinvasion of the CT and disorganization of the CT with dysplastic changes.

## Discussion

The development of an ideal animal model for cancer initiation and progression has been reviewed specially regarding the chemically induced oral cancer<sup>(21)</sup> (22, 23). Salley *et al.*,<sup>(24)</sup> was the first to successfully induce squamous cell carcinoma in the hamster cheek pouch by painting (polycyclic hydrocarbon 9, 10 dimethyl-1, 2, benzanthracene (DMBA) dissolved in benzene or acetone. This was followed by several trials using chemical induction, tumor transplantation, transgenic models and no-carcinogenesis without reaching a true "ideal animal" model. The oldest and least sophisticated method remains the most successful to reproduce the closest human situation<sup>(9)</sup>. DEN is a potent carcinogenic nitrosamine present in tobacco smoke and other food and pharmaceutical agents and has been known to induce hepatocellular carcinoma<sup>(25, 26)</sup>. Our findings indicate that it is a suitable agent for inducing oral carcinogenesis since all animals subjected to it ultimately developed invasive carcinoma evident by epithelial invasion of the basement membrane. This agrees with Haradaa *et al.*,<sup>(27)</sup> who found that DEN induced oral leukoplakic lesions and costochondral hyperplasia in hamsters similar to human leukoplakia.

Garlic is traditionally used as a spice and is known for its medicinal properties and varied pharmacologic functions including antimicrobial, antithrombotic, hypolipidemic and anticarcinogenic effects<sup>(28-35)</sup>. Epidemiologic studies have also provided evidence that increased intake of garlic is associated with decreased cancer risk<sup>(36)</sup>. The anticancer properties of garlic were detected mainly for the aged garlic extract (AGE)<sup>(37)</sup>. In our study, AGE was seen to confer a protective role to the animals when given before the cancer is induced but not to the extent of preventing the induction of cancer at the dose of the DEN used in the study. The animals given AGE before the DEN injections resisted the weight loss observed in the other animals and the clinical features of their lesions appeared less aggressive. A few

observational studies discussed the effects of garlic on oral cancer incidence but according to Kim *et al.*,<sup>(38)</sup> only one was rated as having high methodological quality. Moreover, in accordance to our findings, Geleone *et al.*,<sup>(39)</sup> reported that high use of garlic was associated with a reduction in oral cancer. This study suggests a possible protective role for AGE with the resistance to oral cancer induction such as that previously with colon cancer<sup>(35)</sup>, where AGE was found to decrease cell proliferation and the initiation period of the carcinogenesis.

## Conclusion

Although the AGE garlic in this study showed slower initiation of dysplastic changes however, a larger sample study is required with the use of varying doses of DEN to further clarify this process in an animal model.

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