Histopathological study on Inhibitory effects of *Brassica oleracea* L. Var. Italica (Broccoli) Ethanolic extract on 4-Nitroquinoline-1-oxid -induced Pre-neoplastic changes in the Rat

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**ABSTRACT**

Oral squamous cell carcinoma is one of the most common cancers in the world. Novel treatment is best studied in animal models that mimic the same clinical features as human squamous cell carcinomas. For this end, 48 male Wistar rats were randomly allocated into four groups of 12 animals each. Group 1 served as control and was given the basal diet and tap water without 4-NQO. Rats from Groups 2 to 4 assigned to receive 30 ppm 4-NQO in drinking water for 12 weeks. When the feeding of 4-NQO was started the rats of groups 3 and 4, received broccoli extract at a dose of 200 and 300 mg/kg respectively, 3 times per week. At the end of experiment, the rats were euthanized and the tongue was removed. Tissue specimens collected from tongues and 5 μm thick microscopic sections were prepared through hematoxylin-eosin staining method. Histological evaluations for carcinogenesis were performed for tongues epithelial tissue. The incidences of tongue lesions were compared among the groups. There were no pathological alterations in control rats. Premalignant lesions appeared after 12 weeks of the last application of 4NQO. Administration of broccoli extract at both doses during the experiment caused a significant reduction in the frequency of tongue preneoplasms. The incidences of tongue severe dysplasia in the high dose group was significantly smaller than the low dose group (p<0.05). These findings suggest that broccoli, is effective in inhibiting the development of oral mucosa neoplasms induced by 4-NQO.

**Key words:** *Brassica oleracea* L., 4-Nitroquinoline-1-oxid, Oral carcinogenesis, Rat

**INTRODUCTION**

Oral cancer accounts for almost 275,000 new cases worldwide each year [1]. The main risk factors for developing oral cancer are tobacco smoking and consumption of alcohol [2]. Continuation of smoking during treatment can lead to increased morbidity and mortality [3]. Primary treatment is associated with high morbidity and function loss of the organ, while up to 50% die due to recurrence [4]. Present-day treatment, surgery, radiotherapy or chemotherapy or a combination of these three, has had notwithstanding the improvements, no significant improvement in survival [5]. However, there has not been any kind of long-term treatments reporting success in preventing second primary cancers. New therapies can be investigated both in vitro and in vivo. The drawback of in vitro laboratory study is the dissimilarity between the cell culture and the physiological processes giving misleading results. Several animal models for oral squamous cell carcinoma are used including hamster, rat and mouse models, with each model having its own advantages and disadvantages [6, 7].

In a large number of epidemiological data on the relationships between diet and cancer, a protective effect of the consumption of vegetables and fruits on various forms of cancer including oral cancer is found [8-10].

In fact, several natural compounds present in fruits and vegetables are reported to inhibit chemical carcinogenesis [11-16]. Thus, vegetables and fruits are rich sources for chemopreventive agents against certain cancers [15,17,18]. Recent studies indicate that some flavonoids in daily diets have an inhibitory effect on oral carcinogenesis [13,19,20, 15, 21, 22].
Cruciferous vegetables, belong to family cruciferae in particular of the Brassica genus such as broccoli, cauliflower, kale and brussels sprouts, have significant cancer preventive effects, as shown in epidemiological and animal carcinogenesis studies [23]. They contain substantial quantities of isothiocyanates (mostly in the form of their glucosinolate precursors) some of which (e.g., sulforaphane or 4-methyl sulfinyl butyl isothiocyanate) are very potent inducers of phase 2 enzymes [24]. *Brassica oleracea* L. var. italica (broccoli) is a good source of health promoting compounds since it also contains a variety of polyphenolics [25]. The cancer protective properties of broccoli consumption are most likely mediated through bioactive compounds that induce a variety of physiologic functions including acting as direct or indirect antioxidants, regulating enzymes and controlling apoptosis and the cell cycle [26]. Broccoli also contains other protective constituents like beta-carotene, vitamin C and vitamin E, which can help to reduce reactive oxygen species level and prevent cancers [27]. Broccoli showed its antioxidant and cytoprotective efficacy against many diseases such as parkinson's disease [28], breast cancer [29], bladder cancer [30], prostate cancer [31], lung cancer [32], renal cancer [33], hepatic cancer [34], skin cancer [35], brain injury [36] and cholesterol [37].

In this study, the preventive efficacy of broccoli ethanolic extract (EEB) was evaluated in a 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis model. 4-NQO was used as a carcinogen because it is known to produce a spectrum of preneoplastic and neoplastic lesions in the oral cavity [19, 38]. Of particular interest is that oral lesions induced in rats by oral administration of 4-NQO in drinking water have been reported to be similar to lesions in human [39, 40].

### MATERIALS AND METHODS

#### Animals

The study was conducted in male Wistar rats, aged 10 weeks, with an average weight of 220 g, provided by Central Animal House Facility of Drug Applied Research Center-Tabriz University of Medical Science. A prior approval was obtained from the Animal Ethics Committee of Islamic Azad University of Tabriz (IAUT) for the study protocol. The animals were maintained under the standard conditions of humidity, temperature (25±2°C) and light (12 h light/12 h dark), and fed with commercial pellet diet and water ad libitum.

#### Plant extract and Chemicals

Plant material, *Brassica oleracea* L. var. italica, (broccoli) was purchased from the local market, Tabriz, Iran and authenticated by the Pharmacognosy Department of Tabriz University of Medical Science. Ethanolic extract of broccoli (EEB) was prepared by soxhlet method using 500 ml ethanol (95%) for 100 g (dry weight) of plant material. Extract was concentrated in water bath to semisolid form. The yield of extract was 19.80%. Chemical profile of broccoli extract has been described by various researchers and main identified constituents are glucocynolates, tocopherols, carotenoids, polyphenolics, etc. [56].

4-Nitroquinoline-1-oxid (4NQO) obtained from Sigma Aldrich Co., St. Louis, MO, USA.

#### Study design

48 male Wistar rats were randomly allocated into four groups (1-4) of 12 animals each. Group 1 served as control and was given the basal diet and tap water without 4-NQO. Rats from Groups 2 to 4 assigned to receive 30 ppm 4-NQO in drinking water for 12 weeks. When the feeding of 4-NQO was started the rats of groups 3 and 4, received EEB at a dose of 200 and 300 mg/kg/b.w. in intraperitoneal rout [57] respectively, 3 times per week.

At the end of experiment, the mean body weights of the rats were calculated in order to evaluate the potential toxicities on body weights of rats. The animals were euthanized and the tongues were removed. Tissue specimens collected from tongues, were fixed in 10% buffered formalin, embedded in paraffin and 5 μm thick microscopic sections were prepared through hematoxylin-eosin staining method. Histological evaluations for carcinogenesis were performed for tongues. Epithelial lesions of the tongue were diagnosed according the criteria described by Baonczcy and Csiba [41], Kramer et al. [42] and Schoop et al [6].

#### Statistical analysis

Statistical analysis on the incidence of lesions was performed using Fisher’s exact probability test or Chi-square test and the data including body weight were compared by ANOVA test and Tukey post-hoc test. The results were considered statistically significant if the P value was 0.05 or less.

### RESULTS

The mean body weights at the end of the study are indicated in Table 1. The mean body weights of rats in group 2, 3 and 4 were significantly lower than that of group 1 (p<0.05). There were no significant differences among the groups 2-4 from this view point. In the present study, after 12 weeks treatment, hyperplasia and three types of dysplasia (mild, moderate and severe dysplasia), that are considered to be
preneoplastic lesions for oral cancer were present in the tongue of rats in group 2 through 4, but not in rats of group 1. Almost all rats in group 2 had hyperplasia and all types of dysplasia. Tongues in group 4 did not show severe dysplastic changes although at the same time hyperplasia without atypia and mild to moderate dysplastic changes were detected. These incidences were significantly smaller than those of group 2, with statistical differences between groups 2 and groups 1, 4 and 3 ($p<0.001$, $p<0.01$ and $p<0.05$, respectively). The frequencies of hyperplasia and dysplasia in group 3 were significantly lower than in group 2 ($p<0.05$). However, only two rats from group 4 (given EEB 300 mg/kg during 4-NQO administration) had moderate dysplastic changes. The incidences of such lesions are listed in Table 2 and Table 3.

Microscopically, no histological changes in tongue base epithelia were observed in the control group (Figure 1a). In spite of severe epithelial hyperplasia of tongue, evidences of severe dysplasia in tongue base epithelia as a preneoplastic change was identified in group 2 (4-NQO alone) (Figure 1b). Loss of polarity of epithelium and development a number of small cell nests in spinous layer with primary keratin pearl formation were found in rats with severe dysplasia (Figure 2a). Moreover, formations of severe exophytic hyperplasia in epithelial tissue of tongue tip were found in group 2 (Figure 2b). In addition to moderate to severe hyperplasia and hyperkeratosis throughout the whole thickness of tongue epithelia, moderate to severe dysplasia of tongue base epithelia was also found in group 3 (4-NQO +200 mg/kg EEB), (Figure 1c). The Mild to moderate histological changes including hyperplasia, and hyperkeratosis with thickened spinous cell layer was evidenced after 12 weeks treatment in tongue base epithelia of group 4 (4-NQO +300 mg/kg EEB). In this group, tongue base epithelial dysplasia was mainly found in mild to moderate forms (Figure 1d).

Table 1 - Mean body weights in experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>224.38±15.52a</td>
</tr>
<tr>
<td>2</td>
<td>4-NQO alone</td>
<td>193.67±10.15b</td>
</tr>
<tr>
<td>3</td>
<td>4-NQO+ EEB 200 mg/kg</td>
<td>201.33±12.63b</td>
</tr>
<tr>
<td>4</td>
<td>4-NQO+ EEB 300 mg/kg</td>
<td>207.56±14.28b</td>
</tr>
</tbody>
</table>

Different superscripts shows significant differences by ANOVA test (followed by Duncan posttest) ($P<0.05$)

Table 2 - Incidence of tongue preneoplastic changes of rats given 4-NQO plus EEB

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Normal</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>12/12a</td>
<td>0/12a</td>
<td>0/12a</td>
</tr>
<tr>
<td>2</td>
<td>4-NQO alone</td>
<td>0/12</td>
<td>12/12</td>
<td>10/12</td>
</tr>
<tr>
<td>3</td>
<td>4-NQO+ EEB 200 mg/kg</td>
<td>2/12c</td>
<td>10/12c</td>
<td>7/12c</td>
</tr>
<tr>
<td>4</td>
<td>4-NQO+ EEB 300 mg/kg</td>
<td>4/12b</td>
<td>8/12b</td>
<td>5/12b</td>
</tr>
</tbody>
</table>

Different superscript shows significantly different from group 2 by Fisher’s exact probability test (a $P<0.001$, b $P<0.01$ and c $P<0.05$).

Table 3 - Incidence of tongue dysplasia in rats given 4-NQO plus EEB

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats with dysplasia</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mild dysplasia</td>
<td>Moderate dysplasia</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>0/12</td>
<td>0/12b</td>
</tr>
<tr>
<td>2</td>
<td>4-NQO alone</td>
<td>10/12</td>
<td>2/12</td>
</tr>
<tr>
<td>3</td>
<td>4-NQO+ EEB 200 mg/kg</td>
<td>7/12</td>
<td>2/12</td>
</tr>
<tr>
<td>4</td>
<td>4-NQO+ EEB 300 mg/kg</td>
<td>5/12</td>
<td>3/12b</td>
</tr>
</tbody>
</table>

Different superscript shows significantly from group 2 by Fisher’s exact probability test (a $P<0.001$, b $P<0.01$ and c $P<0.05$).
Figure 1- Histologic appearance of tongue epithelium of rats 12 weeks after starting the experiment (H&E staining, Original magnification ×40). (a: control group) Normal tongue with no histological changes. (b: 4-NQO) Severe dysplasia and hyperkeratosis. (c: 4-NQO+200 mg/kg EEB) Moderate dysplasia and hyperkeratosis. (d: 4-NQO+300 mg/kg EEB) Mild dysplasia and hyperkeratosis.

Figure 2- Histologic appearance of tongue epithelium of rat 12 weeks after starting the treatment with 4-NQO (H&E staining). a: Loss of polarity of epithelium and development a number of small cell nests in spinous layer with primary keratin pearl formation (Original magnification ×250). b: Development of exophytic hyperplasia in epithelial tissue of tongue tip (Original magnification ×10).

DISCUSSION
Significant reductions in body weights of the rats in the treatment groups compared to control rats and no statistical difference in the body weights of rats among them with pre-neoplastic lesions at week 12, indicates that administration of EEB at these dosages have no side effects on the rats and this alterations is due to pre-neoplastic lesions induced by 4-NQO.
The results in the present study demonstrated that EEB effectively suppressed 4-NQO-induced oral carcinogenesis as revealed by reduction in the incidence of tongue epithelial dysplasia. This may suggest that such a short-term pilot study may be useful for detecting compounds possessing ‘blocking’ chemopreventive property against oral carcinoma. Vegetable and fruits contain a variety of compounds that inhibit mutagenesis and/or carcinogenesis in laboratory animals [44]. Broccoli is rich in several potent anticancer substances such as indolesglucosinolates, beta-carotene [46]. Broccoli is known for containing isothiocyanates and indoles namely indole-3-carbinol (I3C) are phytochemicals that are well-known protectors against the development of cancer suggesting that greater intakes of these vegetables may lower the risk of several types of cancer including Bladder cancer, Prostate cancer, Breast cancer, Non-Hodgkin’s lymphoma. It also contains lutein, another phytochemical with health benefits [45]. Broccoli contains the compound glucoraphanin, leading to an anticancer compound sulforaphane [46]. Thus, it is likely that EEB is applicable for human clinical trials, although no or less toxicity of the compound should be confirmed by the bioassay.

The results of the present study indicate that administration of EEB at the dose levels of 200 and 300 mg/kg during 4-NQO-induced oral tumorigenesis could effectively suppress tumour development. Several mechanisms by which EEB exerts its antitumorigenic action in oral carcinogenesis could be considered. Although prevention of carcinogenesis might be due to multiple mechanisms, one way of action of anticarcinogens is an enhancing effect on carcinogen detoxification systems. Fruits and vegetables that elevate tissue phase II enzyme levels in rodents can effectively block experimental carcinogenesis and increase the clearance of drugs in human [43,12, 47]. It is reported that broccoli-related compounds exert anti-carcinogenic effects through modulation of phase II enzymes [48, 49]. Broccoli contains substantial quantities of isothiocyanates (mostly in the form of their glucosinolate precursors) some of which (e.g., sulforaphane or 4-methylsulfinylbutyl isothiocyanate) are very potent inducers of phase 2 enzymes [24]. Other possible mechanism is antioxidant properties of broccoli. The ability of antioxidants to scavenge free radicals and thereby decrease the amount of free radical damage to biological molecules like lipids and DNA may be one of their protective mechanisms [50]. We propose that the additive and synergistic antioxidant activity of phytochemical such as flavonoids, phenols, alkaloids, triterpenoids, steroids, etc, present in Brassica oleracea Italica are responsible for the its potent antitumor activity [51, 52, 53]. Induction of apoptosis and antiproliferative activity of broccoli may also contribute its antitumor effects. The antiproliferative activity of broccoli sprout extracts is associated with induction of apoptosis and cell cycle arrest. It has been shown that broccoli sprout extracts preferentially activate the mitochondria-mediated apoptosis pathway and arrest cells in S and M phases. The latter is associated with down-regulation of cell division cycle 25C (Cdc25C) and disruption of the mitotic spindle assembly [54]. Broccoli sprouts are an exceptionally rich source of sulforaphane [1-isothiocyanato4-(methylsulfinyl) butane], a well-known cancer chemopreventive isothiocyanate [24]. Sulforaphane has since been rigorously and extensively studied in many laboratories [55], and additional chemopreventive mechanisms have been discovered, among which the induction of apoptosis and arrest of cell cycle progression have been shown to occur without cell and tissue specificity.

In summary, administration of EEB significantly inhibited 4-NQO-induced tongue tumorigenesis, in conjunction with reduction in the frequency of dysplastic lesions. Although additional studies on dose-dependent efficacy and the mechanistic basis of inhibition should be done, the results described here indicate that EEB might be candidate for a chemopreventive agent against oral carcinogenesis.

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REFERENCES
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