ABSTRACT

Objective: The present study was conducted to elucidate the potential chemopreventive activity of Geraniol (GOH), on alterations in lipid peroxidation, membrane bound enzymes and glycoconjugates against 4-Nitroquinoline-1-oxide (4NQO) induced rat tongue carcinogenesis. Methods: Male Wistar rats were subjected to carcinogen 4NQO and protective nature of GOH (200mg/kg.b.w) was investigated with reference to lipid peroxidation, membrane bound ATPases (Na+ / K+ ATPase, Ca++ ATPase and Mg++ ATPase) and protein bound carbohydrate components (protein bound hexose, hexosamine, sialic acid and fucose). Results: Oral cancer bearing animal shows increased expression of tongue tissue lipid peroxidation, membrane bound ATPases and glycoconjugates indicating oxidative stress induced by 4NQO. Interestingly, administration of GOH was found to be significantly down regulates these activities. The PAS-staining and Scanning Electron Microscopy studies were done for further confirmation. Conclusion: These finding indicates that the protective activity of Geraniol against 4NQO induced oral carcinogenesis by decreasing oxidative damage.

Keywords: Oral cancer, Geraniol, Glycoconjugates, Lipid peroxidation, 4-Nitroquinoline-1-oxide.

INTRODUCTION

Oral cancer is a potentially fatal disease with an increasing incidence and an unchanged 5-year mortality rate. Unfortunately, oral cancer is often still late diagnosed, which leads to an increase in the likelihood of functional impairment due to treatment and mortality rate [1]. Moreover, recent studies indicate that long term habits of tobacco chewing, smoking, betel quid chewing and alcohol consumption are strongly attributed to oral carcinogenesis [2]. It proceeds through three distinct phases of initiation, promotion and progression [3]. In several experimental studies, Male wistar albino rats have contributed significantly to the understanding of precancerous and cancerous lesions of oral carcinogenesis [2]. It was previously reported that, GOH inhibits the growth of colon cancer cells [11], melanoma cells [10], hepatoma cells [12] and pancreatic cancer cells [13]. In addition, antioxidant activity of GOH in experimental oral carcinogenesis has also been reported in previous studies [14]. The present study was designed to focus the protective effect of GOH on Lipid peroxidation, membrane bound enzymes, cell surface abnormalities by measuring the status of glycoconjugates and scanning electron microscopic analysis further conforming, the chemopreventive nature of GOH during 4NQO induced oral carcinogenesis in male wistar albino rats.

MATERIALS AND METHODS

Animals

Wistar strain male albino rats, 8 weeks old (140-150g) were purchased from Tamilnadu Veterinary and animal sciences university (TANIVAS), Madhavaram, Chennai, India. The animals were housed in cage under proper environmental conditions and were fed with a commercial pelleted diet (M/S Hindustan Foods Ltd, Bangalore, India) and tap water. All the experiments were designed and conducted according to the ethical norms approved by institutional animal ethical committee guidelines. (IAEC No. 07/02/2011).

Reagents and chemicals

4NQO and Geraniol were purchased from Sigma Aldrich chemicals Pvt Ltd, Bangalore, India. All other chemicals used were of analytical grade, purchased from SRL Chemicals Pvt Ltd, Mumbai, India.

Experimental design

The experimental animals were divided into four groups, each groups comprising of six animals.

Group 1 Control animals treated with corn oil thrice a week orally for 20 weeks.

Group 2 Oral carcinoma was induced by administration of 50ppm 4NQO dissolved in drinking water for 20 weeks (4NQO alone).

Group 3 Animals were treated with GOH (200 mg/Kg b.wt) dissolved in corn oil thrice a week orally. GOH treatment was started one week prior to the first dose of 50 ppm 4NQO administration (as in group 2) and continued till end of the experimental period (4NQO + GOH).

Group 4 Animals were treated with GOH (200 mg/Kg b.wt) dissolved in corn oil thrice a week orally for 20 weeks to assess the cytotoxicity if any, induced by GOH, and rats were referred as drug control (GOH alone).
After the experimental period, the rats were fasted overnight and anesthetized using diethyl ether and sacrificed by cervical decapitation. A portion of tongue was used for histopathological and scanning electron microscopic (SEM) studies, remaining tissue was homogenized in 0.1 M Tris-HCl buffer pH-7.4 and centrifuged. The supernatant was used for biochemical studies, and total protein in serum and tissue homogenate was done [15]. LPO was assayed [16], with malondialdehyde (MDA) release serving as the index of LPO.

Scanning electron microscopic (SEM) studies

The tongues of experimental rats were fixed with modified Karnovsky solution containing 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. After fixation, the samples were dehydrated in a series of ethanol, immersed in isomyl acetate. Sample were cut into small part and placed on the stab and put into E-1010-Hitachi ion coater for carbon coating. After coating, the samples were observed by S-3400N-Hitachi scanning electron microscope and photomicrographs were taken.

Determination of membrane-bound enzymes

Na+/K+ ATPase activity was determined [17]. The activity of Ca2+ ATPase [18] and Mg2+ ATPase [19] was assayed.

Assay of glycoproteins

Hydrolysis of glycoprotein for the determination of hexose, hexosamine and fucose was carried out. A known amount of defatted tissue was put into a test tube to which 1 ml of 2N HCl was added, and the tubes were sealed. Hydrolysis was completed by keeping the sealed tubes at 100°C for 16-18h. After hydrolysis, the contents were neutralized with NaOH and made up to known volume, and aliquots were used for hexose, hexosamine and fucose determination [20]. Sialic acid was determined by the method of Warren [21].

Histopathology

For histopathological examination, Rat tongue tissue were fixed in 10% formalin and routinely processed and embedded with paraffin, 2–3 μm sections were used for histological studies. For detection of glycoconjugates, the tissue sections of tongue were immersed in a solution of 0.1% periodic acid for 15 minutes, at 50°C. The slides were washed in running tap water and immersed in Schiff's reagent for 40 minutes. Subsequently, the sections were washed in running tap water for 10 minutes, counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene and mounted in resinous medium.

Statistical analysis

All the grouped data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Duncan’s multiple range test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± S.D for six animals in each group.

RESULTS

Scanning electron microscopic analysis

Scanning electron microscopic analysis of the control group 1 (Figure 1A) showed numerous sharp conical projections of filiform papillae with uniform keratinized tips arranged in parallel rows depicting constant antero-posterior direction towards the tongue root. The covering epithelial cells appeared with smooth linear edges. Whereas, 4NQO induced rat tongue shows completely disorganized distribution of filiform papillae which appear severely destroyed with desquamation of its epithelial covering and loss of keratinized tips (Figure 1B). GOH treated rat tongue shows normal filiform papillae with regular distribution and inclination, while some of them depict blunt and serrated tips (Figure 1C). GOH alone treated group 4 animals shows almost normal direction and inclination of filiform papillae as control group signifying the cytoprotective effect of GOH (Figure 1D).

Fig. 1: Scanning Electron Microscopy of tongue of control and experimental group of animals.

(A) Group 1- control tongue shows numerous sharp conical projections of filiform papillae with uniform keratinized tips arranged in regular rows. (B) Group 2- 4NQO induced oral cancer bearing animal shows complete loss of filiform papillae and flattened surface. (C) Group 3- 4NQO+GOH treated tongue shows blunt and serrated tips, with rough keratinized cells. (D) GOH alone treated tongue shows normal direction and distribution of filiform papillae.
Effect of GOH on Lipid peroxidation in Tongue and Erythrocyte membrane of control and experimental animals

The levels of LPO in the tongue and erythrocyte membrane of experimental animals in Figure 2. A highly significant (P<0.05) increase in the levels of LPO measured in terms of malondialdehyde were observed in group 2 cancer bearing animals when compared to group 1 control animals. On the contrary, the GOH treatment normalized the levels of tongue and erythrocyte LPO in group 3 animals. However, there was no noticeable change observed in Group 4 GOH alone treated animals when compared to group 1 control animals.

The effect of GOH administration on the activity of tongue and erythrocyte ATPases in the control and experimental groups

The activity of tongue and erythrocyte ATPases, such as Na\(^+/K^+\) ATPase, Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase of control and experimental animals are presented in Table 1 and Figure 3, respectively. A significant decrease in the levels of Na\(^+/K^+\) ATPase, Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase were observed in group 2 cancer bearing animals when compared to group 1 control animals. Pretreatment of GOH to 4NQO treated rats normalized the levels of tongue and erythrocyte ATPases in group 3 animals. However, there was no significant change in Group 4 GOH alone treated animals when compared to group 1 control animals.

Fig. 2: It shows the Level of malondialdehyde (MDA) in tissue and erythrocyte membrane of control and experimental rats.

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P < 0.05 compared with *group 1, †group 2, and ‡group 3 based on Duncan’s multiple range tests.

Table 1: It shows the effect of GOH administration on the activity of ATPases of tongue tissue in the control and experimental groups

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I Control</th>
<th>Group II 4NQO</th>
<th>Group III 4NQO+GOH</th>
<th>Group IV GOH alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+/K^+) ATPase</td>
<td>6.1 ± 0.26</td>
<td>3.16 ± 0.3</td>
<td>4.46 ± 0.4</td>
<td>6.16 ± 0.52</td>
</tr>
<tr>
<td>Mg(^{2+}) ATPase</td>
<td>11.28 ± 0.37</td>
<td>7.28 ± 0.3</td>
<td>8.11 ± 0.23</td>
<td>11.25 ± 0.44</td>
</tr>
<tr>
<td>Ca(^{2+}) ATPase</td>
<td>10.2 ± 0.55</td>
<td>5.58 ± 0.34</td>
<td>7.25 ± 0.38</td>
<td>10.18 ± 0.5</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D for six rats in each group. Statistical significance p < 0.05 compared with *group 1, †group 2, and ‡group 3 based on Duncan’s multiple range test. Units: Na\(^+/K^+\) ATPase, Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase are expressed µmol of inorganic phosphate formed / min (mg /protein).

Fig. 3: It shows the Level of erythrocyte membrane ATPases such as Na\(^+/K^+\) ATPase, Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase of control and experimental animals.

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P < 0.05 compared with *group 1, †group 2, and ‡group 3 based on Duncan’s multiple range tests.
The effect of GOH administration on cell surface glycoconjugates abnormalities

Figure 4 and Table 2 shows the levels of glycoconjugates (protein bound hexose, hexosamine, total sialic acid and fucose) in the plasma and tongue of control and experimental rats in each group, respectively. The levels of glycoconjugates in the plasma and tongue tissue were significantly increased in animals treated with 4NQO alone (group 2) as compared to control (group 1) animals. Oral administration of GOH to 4NQO treated rats (group 3) brought back the levels of above said glycoconjugates to near normal range. No significant difference was noticed in the levels of plasma and tongue glycoconjugates in GOH alone (group 4) treated animals as compared to control (group 1).

Figure 5 (A - D) shows glycoconjugates expression pattern in the tongue of control and experimental rats in each group. The glycoconjugates expression pattern was analyzed using periodic acid Schiff's staining in the tongue tissue. We observed increased glycoconjugates expression in the tongue of tumor bearing group 2 animals (Figure 5B). Oral administration of GOH to 4NQO treated group 3 animals (Figure 5C) significantly reduced the expression of glycoconjugates in the tongue tissue. Glycoconjugates expression pattern was similar in GOH alone treated group 4 animals, when compared to (Figure 5D) and control group 1 animals (Figure 5A).

**Fig. 4:** It shows the Level of glycoconjugates such as protein bound hexose, hexosamine, total sialic acid and fucose in the plasma of control and experimental rats

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P < 0.05 compared with a group 1, b group 2, and c group 3 based on Duncan’s multiple range tests.

**Table 2:** It shows the effect of GOH on tissue glycoprotein’s like hexose, hexosamine, total sialic acid and fucose in the control and experimental groups

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I (Control)</th>
<th>Group II (4NQO)</th>
<th>Group III (4NQO+GOH)</th>
<th>Group IV (GOH alone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose</td>
<td>92.08 ± 3.1</td>
<td>114.83 ± 4.02</td>
<td>103 ± 3.27</td>
<td>91.93 ± 2.9</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>35.2 ± 3.8</td>
<td>71.02 ± 6.9</td>
<td>43.5 ± 4.1</td>
<td>34.8 ± 3.6</td>
</tr>
<tr>
<td>Total sialic acid</td>
<td>14.83 ± 3.4</td>
<td>26.33 ± 2.5</td>
<td>21.16 ± 1.7</td>
<td>15.83 ± 3.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>12.5 ± 1.87</td>
<td>20.16 ± 2.7</td>
<td>17.5 ± 1.8</td>
<td>12.33 ± 1.3</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D for six rats in each group. Statistical significance p < 0.05 compared with a group 1, b group 2, and c group 3 based on Duncan’s multiple range test. Units: Hexose, hexosamine, total sialic acid and fucose units are expressed mg/g tissue protein.

**Fig. 5:** It shows Glycoconjugates expression pattern in the tongue of control and experimental animals in each group (40X).

(A) Normal glycoconjugates expression in the control rats. (B) 4NQO administered oral cancer bearing rat tongue section indicates (black arrow) over expression of glycoconjugates ( ). (C) Lowered expression of glycoconjugates in 4NQO+GOH treated animals. (D) Normal glycoconjugates expression in GOH alone treated animals.
DISCUSSION

The search for new chemopreventive and antitumor agents that are both effective and less toxic has kindled great interest in phytochemicals [22]. Geraniol, an acyclic monoterpene found in lemon, lemongrass and aromatic herb oils. It has been shown to inhibit the free radical formation and reduced tumor incidence. Lipid peroxidation is one of the major mechanisms of cellular injury caused by free radicals [23]. Administration of 4NQO has been reported to generate lipid peroxidation products and induce oxidative damage by converting 4-hydroxyaminoquinoline-1-oxide (4HAQO), which is the alternative carcinogen in 4NQO metabolism [24]. The level of LPO increases with the administration of 4NQO during oral carcinogenesis, which leads to impairment in the lipid architecture of cellular membrane and increase membrane damage [25]. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense [26]. From the present study, it is evident that an increased level of LPO was found in cancer bearing animals. However, the administration of GOH decreased the LPO level, which may be due to the free radical scavenging activity.

In malignancy, the cell membrane plays a crucial role in the stimulation and control of cell adhesiveness, mortality and proliferation [27]. The protection of membranes is of potential importance in the treatment of disease processes. The membrane bound enzymes such as Na+/K+ ATPase, Mg2+ ATPase and Ca2+ ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP by hydrolysis[20,29]. Na+/K+ ATPase activity is responsible for a large part of the energy consumption that constitutes the basic metabolic enzyme activity and indicates a change in the membrane under special nutritional [30] or pathological conditions. Ca2+ ATPase is a reflection of energy dependent calcium transport across the cell membrane. Decreased activity of this enzyme would contribute to the accumulation of intracellular calcium, which will then bind to the inner surface of the membrane and make the membrane less deformable [31]. Mg2+ ATPase, along with the other ATPase is also involved in energy requiring process in the cell [32]. In addition, peroxidation of membrane lipid initiates a loss of membrane bound enzyme activity and alters membrane permeability and cell function [27]. In the present study, we observed reduced activity of all the three membrane bound ATPases namely Na+/K+ATPase, Mg2+ATPase and Ca2+ATPase in 4NQO induced oral cancer bearing animals. Whereas GOH treatment returned the activities of these enzymes to near normal level. This shows protective effect of GOH by either due to scavenging peroxides before attacking membrane or due to blocking the oxidation of membrane lipids. Neoplastic transformation is associated with altered cell surface carbohydrate composition of the cell membrane and the changes in the surface of tumor cells are relevant to their abnormal growth, metastasis and changes in cell adhesion [33]. Aberrant glycosylation is the key feature involved in the conversion of normal cell into a malignant one. Atypical glycosylation and degradation of cell surface carbohydrates have been shown in oral carcinogenesis [34]. A large number of experimental studies pointed out that glycoproteins were synthesized enormously during cancerous conditions and subsequently entered into circulation [35]. Over expression of glycoconjugates in the tumor cells with subsequent shedding into plasma could account for increased levels of plasma protein bound hexosamine, hexosamine sulfate and fucose. Elevated levels of these proteins have been reported in the plasma of various cancer patients [36]. Evidence from animal experiments suggests that the presence of malignant tumors invoke increased synthesis of glycoproteins, which subsequently enter into the circulation [37]. These findings agreed with our present observation where we noticed an increase level of tissue and serum glycoconjugates in oral cancer induced animals. GOH pretreatment significantly reduced the level of these glycoproteins to near normal. These reductions in the levels of these glycoprotein components indicate that GOH (20mg/Kg b.w.) has the ability to suppress malignancy by modulating cellular transformation and protects the cell wall abnormalities.

CONCLUSION

Geraniol significantly attenuated the hazardous consequences of 4NQO induced oral carcinogenesis via modulating lipid peroxidation, cell surface glycoconjugates and membrane bound ATPases probably through its strong antioxidant and anticancer activity which ultimately render protection to the membrane from deleterious effect of free radicals which were induced by carcinogen 4NQO as shown in the schematic diagram (Figure 6). Additionally, the Scanning electron microscopic analysis further confirming, the protective nature of GOH against 4NQO induced oral carcinogenesis. However, studies are in progress to explore the mechanism by which GOH prevents experimental oral carcinogenesis.

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