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Modulatory efficacy of Green tea polyphenols on glycoconjugates and immunological markers in 4-Nitroquinoline 1-oxide-induced oral carcinogenesis—A therapeutic approach

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Abstract

Green tea polyphenols (GTP) has been used as a chemopreventive agent world wide against chemically induced cancer. The present study is aimed to understand the therapeutic action of GTP on glycoconjugates and immunological markers in 4-Nitroquinoline 1-oxide (4-NQO)-induced oral cancer over a period of 30 days at 200 mg/kg, *p.o.*, Oral cancer was induced by painting 4-NQO for 8 weeks followed by administration of GTP after 22 weeks, for 30 days. Glycoconjugates such as hexose, hexosamine, sialicacid, fucose and mucoprotein were analysed. Expression of glycoconjugates was examined through histology and SDS-PAGE. Immunological markers such as circulating immune complex and mast cell density were studied. Oral cancer-induced animals showed a significant increase in levels of glycoconjugates and its expression, similar to that observed for immunological markers. Treatment with GTP altered the expression of glycoconjugates as well as immunological markers. The results suggest that GTP modulates both the expression of glycoconjugates and immunological markers resulting in regression of oral cancer.

Keywords: Oral cancer; 4-Nitroquinoline 1-oxide; Green tea polyphenols; Glycoconjugates; Immunological markers; Mast cell

1. Introduction

The significance of glycoproteins in bringing about neoplastic transformation is well known. Glycoconjugates are essential for the assembly of the oligosaccharide moieties of the glycoprotein chains and their levels have been found to be elevated in neoplastic conditions and can therefore be designated as non-specific markers of malignancy [1]. Glycoprotein levels are high in tumour tissue due to increased lipid peroxidation resulting in lowered antioxidant status [2], aberrant glycosylation [3] and increased lysosomal hydrolases and proteases [4].

In experimental oral cancer, the activity of lysosomal enzymes such as β-D-galactosidase, β-D-glucuronidase and cathepsin D were reported to be markedly increased [4]. The activity of phospholipase C and A_2 was found to be elevated in oral cancer [5]. These increase in phospholipase and lysosomal enzymes may contribute to the increase in glyconjugates.

To elucidate the role of angiogenesis in carcinogenesis and progression of oral cancer, we investigated mast

Abbreviations: g, grams; rpm, revolutions per minute; 4-NQO, 4-nitroquinoline 1-oxide; GTP, Green tea polyphenols; MC, mast cell; MCD, mast cell density

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cell density on a paraffin-embedded pathological tissue. Toluidine blue histochemical method was employed for mast cell identification [6].

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck, with a worldwide incidence of over 300,000 new cases annually. It is characterized by a high rate of morbidity and mortality (over 50%) and in that aspect is similar to malignant melanoma [7,8]. Incidence of oral cancer varies widely between countries and geographical areas of the world [9], and is generally most common in developing countries. These variations have traditionally been explained by the exposure of these populations to specific risk factors, such as tobacco and alcohol [10].

4-NQO is known to induce multistep carcinogenesis [11] and serves as a good model to investigate oral carcinogenesis. The carcinogenic potential of 4-NQO is now well documented, and it has been shown to produce oral squamous cell carcinoma as well as spindle cell sarcoma in various rodent species [12]. Carcinoma is preceded by a sequence of hyperplasia–papilloma/dysplasia–carcinoma, similar to that of human oral cancer. 4-NQO is also known to induce H-ras mutation in chromosome 7 leading to head and neck squamous cell carcinoma in experimental murine model, which is quite similar to that of tumors that develop in tobacco chewers [13].

Green tea is a popular beverage consumed in some parts of the world and is a rich source of polyphenols, which are antioxidant in nature [14]. Green tea contains many polyphenols known as catechins, including epigallocathechin-3-gallate (EGCG), epigallocatechin (EGC) and epicathechin-3-gallate (ECG) [15]. In recent years, experimental studies have provided growing evidence for the beneficial action of GTP on multiple cancer-related biological pathways (carcinogen bioactivation, cell-signaling, cell cycle regulation, angiogenesis, oxidative stress, inflammation). Although the epidemiologic data on GTP and cancer are still limited and conflicting, some protective associations have been suggested for GTP [16]. Epidemiological studies have associated the consumption of Green tea with a lower risk of several types of cancer including stomach, oral cavity, esophagus and lung. In fact, tea is one of the few agents that can inhibit carcinogenesis at the initiation, promotion and progression stages [14]. The worldwide interest in Green tea as a cancer preventive agent for humans has increased owing to its non-toxic, effective nature in a wide range of organs [17].

In the previous study we analyzed cellular thiol status on therapeutic administration of GTP to 4-NQO-induced oral cancer [18]. The present study evaluates the modulating effect of GTP on glycoconjugates and immunological markers in 4-NQO-induced oral cancer.

2. Materials and methods

2.1. Materials

4-NQO was purchased from Sigma Chemical Company (St. Louis, MO, USA). Fresh Green tea leaves were collected from The Nilgris. All other chemicals used were of analytical grade.

2.2. Preparation of GTP

Extraction of Green tea polyphenols (GTP 80%) was done by adapting the procedure of Shaowen Lee (Director, Human King long Bioresource Co. Ltd., China) followed by Srinivasan et al. [18].

2.3. Animals

Wistar strain male albino rats (10 weeks old) weighing 180–200 g were purchased from TANUVAS (Chennai, India). The animals were housed, four per cage in a room with controlled temperature and humidity with 12 h light:dark cycles. All the animals were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and tap water *ad libitum*. This study was conducted as per the guidelines of the animal ethical committee of our institution (IAEC No. 01/12/04).

2.4. Experimental protocol

The animals were divided into 4 groups of 12 animals each. Group 1 (24) served as control, In group 2 (48) oral carcinoma was induced by painting 4-NQO 0.5% in propylene glycol in the oral cavity using no.4 painting brush three times/week for 8 weeks, after 22 weeks oral cancer was induced. Group 3 (24) served as drug control and received 200 mgs of GTP/kg, b.w. oral intubations for 30 days. Group 4 (24 from group 2) served as treated, receiving 200 mgs of GTP/kg b.w. oral intubations for 30 days, from 22 to 26 weeks.

After the experimental period, the animals were anaesthetized using ether, sacrificed by cervical decapitation. The mouth was cut opened using a surgical knife. The tongue was excised out, weighed and the tissues were homogenised in Tris–HCl buffer pH 7.4 and centrifuged at 3000 rpm for 10 min. The supernatant obtained was used for various assays.

A portion of the tongue tissue was fixed in 10% buffered neutral formalin solution for histological studies. Blood was collected and serum was separated for fur-

ther analysis. After fixation, tissues were embedded on paraffin and solid sections were cut at 5 μm and used for histological studies. Protein was estimated by the method of Lowry et al. [19]. Histochemical staining of Glycoconjugates was carried out as per the method of Meloan et al. [20] using 2% periodic acid and Schiff's reagent in dark for 20 mins. Photomicrographs were obtained using a Nikon Y-FL ECLIPSE 400 (Japan) microscope connected to a Nikon FDX-35 camera (Japan) to measure the relative intensity of PAS staining with the aid of a 4×/0.10α/−WD3.0 and 10×/0.25α/−WD6.1 magnification lens in control and experimental groups.

About 12.5% SDS–PAGE was carried by the method of Laemmli [21]. After completion of the electrophoresis run, the gel was removed from the plates and was immersed in 12.5% TCA for 30 min and rinsed in distilled water for 30 s, then immersed in 1% periodic acid in 3% acetic acid for 50 min, washed repeatedly in distilled water for five times then immersed in Schiff's reagent in dark for 50 min. The gel was destained with three changes of 0.5% freshly prepared sodium meta bisulphite solution. It was then stored in 3% acetic acid and the gel was photographed. Simultaneously marker was run along with the samples and stained with coomassive brilliant blue stain for reference.

Hexose was estimated by the method of Niebes [22]. Hexosamine was estimated by the method of Wagner [23]. Sialic acid was estimated by the method of Winzler [24]. Fucose content was estimated by the method of Dische and Shattles, [25]. Mucoproteins were estimated by the method of Winzler [24]. Lysosomal fraction was isolated by the method of Wattiaux [26]. The activity of β-D-glucuronidase was determined by the method of Hultberg et al. [27]. The activity of β-D-galactosidase was assayed by the method of Conchie et al. [28]. Acid phosphatase was assayed by the method of King [29]. Assay of Phospholipase C was carried out by the method of Zwaal et al. [30]. Assay of Phospholipase A_2 was carried out by the method of Clark et al. [31].

Histochemical analysis of mast cell was carried out by the method of Ranieri et al. [6]. Level of serum gamma globulin was estimated by the method of Chauhan [32]. Serum CIC was estimated by the method of [33].

2.5. Statistical analysis

All data were analysed with SPSS/10 Student Software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The values are expressed as mean ± S.D., P value of less than 0.001, 0.01 and 0.05 was considered to indicate statistical significance.

3. Result

Histological staining of glycoconjugates by periodic acid Schiff's staining (PAS) is shown in Fig. 1, which

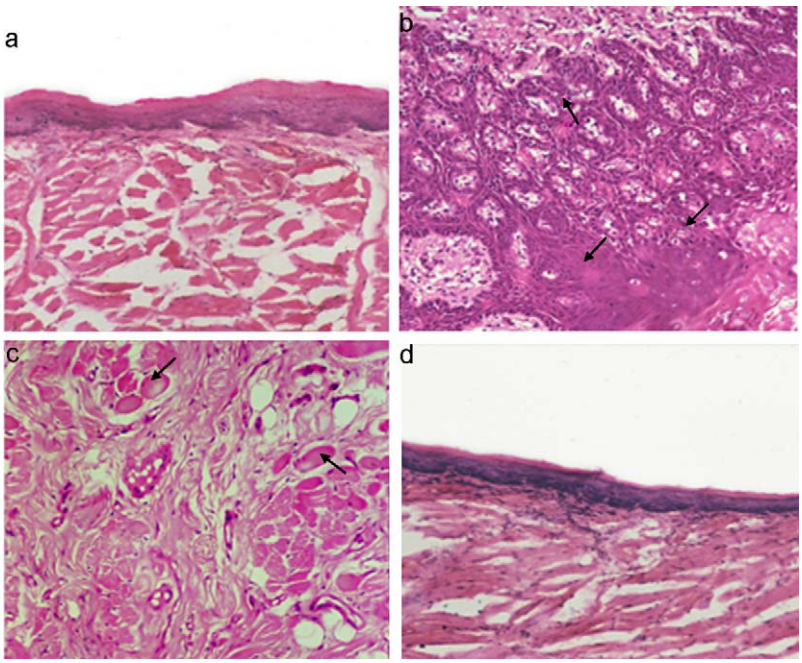

Fig. 1. Histochemical analysis by periodic acid schiffs staining in control and experimental animals. (a) Control, (b) OC-induced, (c) OC-induced GTP treated and (d) GTP alone. → Depicts glycoconjugates. 10×/0.25α/−WD6.1 magnification.

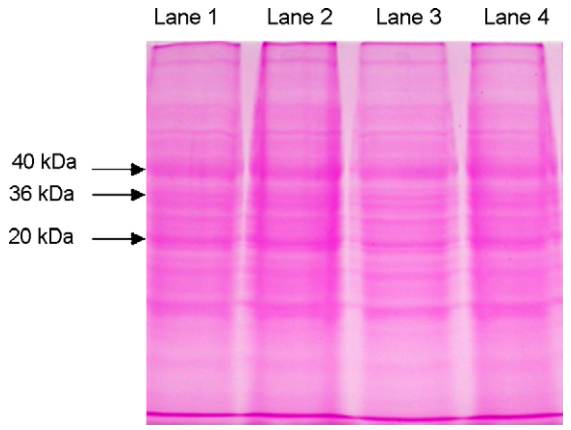

Fig. 2. Glycoconjugates staining by periodic acid schiffs on SDS-PAGE. Lane 1: control; lane 2: OC-induced; lane 3: GTP alone; lane 4: OC-induced GTP treated.

shows a clear increase in glycoconjugate expression in oral cancer-induced animals (b) with high pearl formation in the tongue. GTP treated to oral cancer-induced rats showed a mild decrease in glycoconjugates with degradation of tumor cells (c).

Fig. 2 depicts the expression of glycoconjugates, control (lane 1), oral cancer-induced (lane 2), GTP alone (lane 3) and GTP treated (lane 4). There was an arbitrary increase in the level of glycoconjugates in oral cancer induced (lane 2) when compared to control animals (lane 1). There was a significant decrease in the glycoconjugate level in GTP treated (lane 4) to oral cancer-induced animals when compared to oral cancer-induced animals (lane 2).

Glycoconjugates level in the tongue of control and experimental animals are presented in Fig. 3a and b. Level of glycoconjugates showed a significant ($p < 0.001$) increase in group 2 oral cancer-induced animals when compared with control animals. Group 4 (OC → GTP) rats showed a significant ($p < 0.001$) decrease in glycoconjugates when compared with group 2. No significant changes were found between group 1(control) and group 3(GTP).

In OC-induced rats, there was a significant ($p < 0.001$) increase in the activity of lysosomal enzymes such as β-D-galactosidase, β-D-glucuronidase and acid phosphatase when compared to that of control animals (Fig. 4). Administration of GTP to the rats significantly ($p < 0.001$ and $p < 0.01$) increased the activity of lysosomal enzymes when compared to that of oral cancer-induced rats. No significant changes were observed when control rats are compared with GTP alone treated rats.

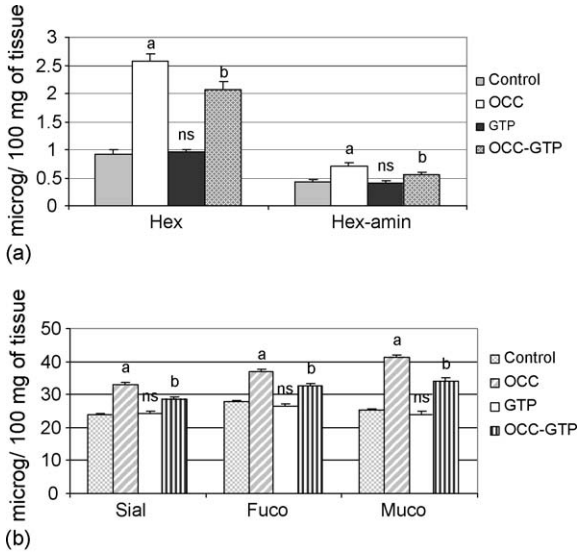

Fig. 3. (a) Effect of GTP on the levels of hexose and hexoseamine in the tongue of control and experimental groups. (b) Effect of GTP on the levels of sialic acid, fucose and mucoprotein in the tongue of control and experimental groups. Values are expressed as mean ± S.D. (Bonferroni's multiple comparison test). Comparisons: control vs. OC and GTP; OC vs. OC-GTP; ns: non-significant; $^{a}P < 0.001$; $^{b}P < 0.01$.

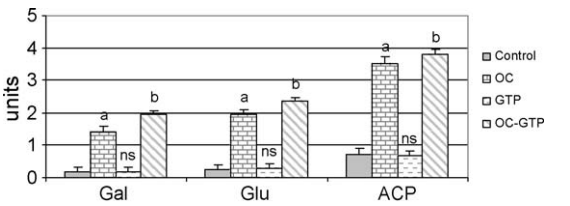

Fig. 4. Effect of GTP on the activities of glucronidase, galacturinadase and ACP in the tongue of control and experimental groups. Values are expressed as mean ± S.D. Units: glucuronidase and galactosidase (mmoles of *p*-nitrophenol liberated/h/100 mg protein). Acid phosphatase (mmoles of phenol liberated/min/100 mg protein). Comparisons: control vs. OC and GTP; OC vs. OC-GTP; $^{a}P < 0.001$; $^{b}P < 0.01$; ns: non-significant (Bonferroni's multiple comparision test).

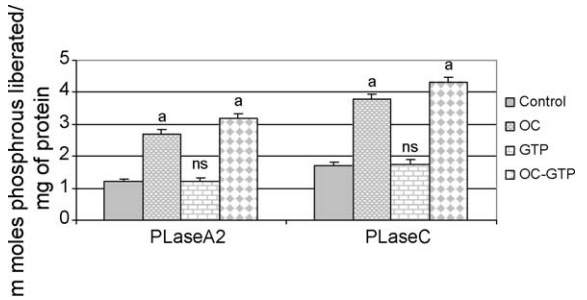

Fig. 5. Effect of GTP on the activities of phospholipases. Values are expressed as mean ± S.D. Comparisons : control vs. OC and GTP; OC vs. OC-GTP; ns: non-significant; $^{a}P < 0.001$ (Bonferroni's multiple comparision test).

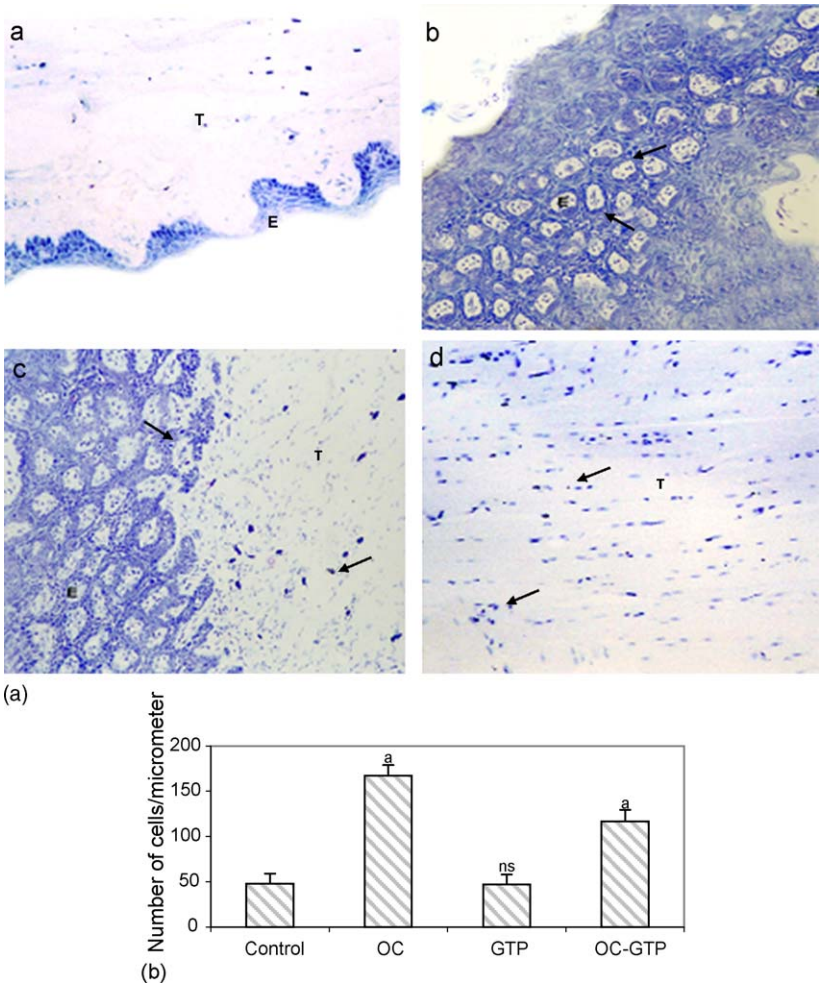

Fig. 6. (a) Histochemical analysis of mast cells in control and experimental animals. (a) Control, (b) OC-induced, (c) OC-induced GTP treated and (d) GTP alone. → Depicts mast cells. 10×/0.25α/−WD6.1 magnification. (b) Mast cell density in control and experimental groups. Values are expressed as mean ± S.D. $P < 0.05$; control vs. GTP are statistically non-significant (ns); control vs. OC and OC vs. OC-GTP are statistically significant (a) by Wilcoxon sign Rank test, Asymp. Sig. (two-tailed); $P < 0.028$.

Fig. 5 represents the activity of phospholipase in the tongue of control and experimental animals. In the present study, there was a significant ($p < 0.001$) increase in the activity of phospholipase C and A_2 in OC-induced animals when compared to control animals. GTP treated to oral cancer-induced animals showed a significant increase when compared to OC-induced animals.

Mast cell population in control and experimental animals is depicted in Fig. 6a and b. Giant mast cells and significant increased population were noticed in OC-induced animals (b) when compared to control animals (a). GTP treated to OC-induced animals (c) showed a significant decrease in giant and total mast cell population when compared to OC-induced-induced animals (b).

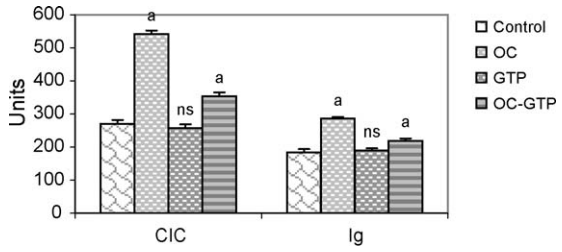

Fig. 7. Effect of GTP on the levels of circulating immune complexes and total serum immunoglobulins of control and experimental groups. Values are expressed as mean ± S.D. Units: CIC ($OD/10^3$); Ig (mg/100 ml). Comparisons: control vs. OC and GTP; OC vs. OC-GTP; $^aP < 0.001$; ns: non-significant (Bonferroni's multiple comparison test).

In the present study, there was a significant ($p < 0.001$) increase in the content of serum immunoglobulins and CIC to that of control animals (Fig. 7). Administration of GTP to oral cancer-induced animals significantly ($p < 0.001$) reduced both CIC and serum immunoglobulin to that of OC-induced rats.

4. Discussion

Extracellular matrix plays an integral role in the biological processes of development, tissue repair and metastasis by regulating cell proliferation, differentiation, adhesion, and migration. Experimental evidence shows that tobacco exposure to human oral cavity alters the glycoconjugates in extracellular matrix that is critical for maintenance, proliferation, differentiation, angiogenesis, and apoptosis of cells [34–36]. Solid tumor growth involves important function such as angiogenesis, which involves stromal components that regulate functions such as cellular adhesion, migration, and gene expression [37].

Betaglycan, chondrition sulfate (∼20–50 kDa) and decorin are the glycoconjugates or stromal components found mostly in the tongue of experimental animals. Betaglycan (∼36 kDa) glycoconjugate is covalently linked to a single side chain of chondrotin sulfate or dermatan sulfate [38]. A small leucine-rich proteoglycan decorin (∼40 kDa) is found associated with negative regulation of cell growth. It has a prominent role in transforming growth factor β and epidermal growth factor receptor activation pathways that contributes to its role in cellular proliferation, angiogenesis, and immunomodulation [37,39].

In the present study there was significant increase in the expression of glycoconjugates in histological examination and protein profile in oral cancer-induced animals. In SDS-PAGE, glycocojugates of ∼40 and ∼36 kDa was found to be expressed more in oral cancer to that of control and drug alone treated rats. There was a significant reduction in the expression in therapeutic administration of GTP to oral cancer-induced group.

In addition to their anti-cancer properties, GTP more particularly EGCG inhibit the binding and efflux of drugs by P-glycoprotein. Thus, GTP or EGCG might be a potential agent for modulating the bioavailability of P-glycoprotein substrates at the intestine and the multidrug resistance phenotype associated with expression of P-glycoprotein in cancer cells [40]. Administration of GTP to oral cancer-induced animals significantly decreased the levels and expression of glycoconjugates, which might be attributed to the modulatory effect of EGCG, one of the catechins in GTP.

In experimental oral cancer, the activity of lysosomal enzymes such as β-D-galactosidase, β-D-glucuronidase, cathepsin D were reported to be markedly increased [4] which well correlated with our study. Administration of GTP to oral cancer-induced animals showed a marked elevation in the activity of lysosomal enzymes, which might be due to extensive tissue break down as reported for therapeutic tumour regression.

In the present study, there was a significant increase in the activity of phospholipase C and A_2 that concurs with the previous study [5]. Green tea catechin improves microsomal phospholipase A_2 activity and the arachidonic acid cascade system in the kidney of diabetic rats [41]. Phospholipase C was found to be activated by EGCG a major polyphenol in GTP [42]. Earlier reports on GTP correlated well with our present study. Treatment with GTP to oral cancer-induced animals drastically increased ($p < 0.001$) the activity of phospholipase C and A_2 in group 4 animals thereby resulting in the utilization of phospholipid content of the cell membrane leading to lysis or necrosis of cancer cell.

The elevation in the activity of lysosomal enzymes and phospholipases resulting in the liberation of glycoconjugates from the tissue, followed by the excess synthesis of the same to compensate for the loss due to liberation can be considered as the possible mechanism for the elevated levels of glycoconjugates upon induction of oral cancer. Therapeutic administration of GTP was found to inhibit phospholipases, which in turn inhibits loss of glycoconjugate and hence excess synthesis. Consequently the levels of glycoconjugates were maintained in GTP treated oral cancer-induced animals.

Mast cells (MCs) have been implicated in promoting angiogenesis in some malignant tumours, especially of the aerodigestive tract, little is known in oral squamous cell carcinoma (SCC). The densities of MCs appeared to increase with disease progression. The MC counts were significantly higher in oral SCC than in normal oral mucosa. MCs may upregulate tumour angiogenesis in oral SCC, perhaps via MC tryptase [43]. Earlier reports on the increase of MC count in oral cancer correlates well with our study; where there was a significant increase in the mast cell population in oral cancer-induced animals. GTP inactivates MC in oral cancer-induced animals thereby significantly reducing mast cell density by blocking the release of histamine [44].

In the present study, there was a significant increase in the content of serum immunoglobulins and CIC to that of control animals. Previous study by Remani et al. [45] states that the circulating immune complexes and their immunoglobulin contents were found to be elevated significantly both in oral submucous fibrosis

and oral cancer. This helps in monitoring the malignant transformation of oral submucous fibrosis to oral cancer. Previous reports on GTP shows a significant delayed production of autoantibodies and IC in GTP-fed MRL-Faslprcg/Faslprcg mice [46] and total IgG [47] was also significantly reduced.

Thus GTP acts immunologically by modulating CIC and serum Ig and attenuates MC activation, which plays a role in angiogenesis and progression of oral cancer. Hence it could be concluded that GTP is a potential candidate in modulating the expression of glycoconjugates and immunological markers there by regressing oral cancer.

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