Evaluation of chemopreventive effects of Thymoquinone on cell surface glycoconjugates and cytokeratin expression during DMBA induced hamster buccal pouch carcinogenesis

G. Rajkamal, K. Suresh*, G. Sugunadevi, M.A. Vijayaanand & K. Rajalingam
Department of Biochemistry & Biotechnology, Faculty of Science, Annamalai University, Annamalai Nagar, Tamilnadu, 608 002 India

The present study aimed to investigate the membrane stabilizing effect of Thymoquinone (TQ) on cell surface glycoconjugates and cytokeratin expression against DMBA induced hamster buccal pouch carcinogenesis. 0.5% DMBA painting (three times per week) in hamster buccal pouches for 14 weeks resulted in the formation of well developed oral squamous cell carcinoma. We observed 100% tumor formation with marked abnormalities of glycoconjugates status in tumor bearing hamsters as compared to control animals. Oral administration of TQ at a dose of 30 mg/kg body weight, to DMBA painted hamsters on alternate days for 14 weeks, reduced the tumor formation as well as protected the levels of cell surface glycoconjugates in DMBA painted hamsters. The present study thus suggests that TQ has potent chemopreventive efficacy as well as protected the abnormalities on cell surface glycoconjugates during DMBA induced hamster buccal pouch carcinogenesis [BMB reports 2010; 43(10): 664-669]

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is part of a group of cancers called head and neck cancers. It can develop in any part of the oral cavity or oropharynx. This form of cancer which includes cancer of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (1). The annual estimated incidence of oral cancer is about 2,75,000 with the majority of these cancers occurring in developing countries like India (2). It has been reported that use of tobacco either in the form of smoking and or chewing is associated with 75-85% of oral cancers (3). The most important risk factor for the development of oral cancer in the Western countries is the consumption of tobacco as well as alcohol (4). Heavy alcohol consumption and smoking are independent risk factors; they have a synergistic effect on oral cancer risk (5). 7,12-dimethylbenz (a)anthracene (DMBA), a potent organ and site specific carcinogen known to induce multistep carcinogenesis, it has been preceded by a sequence of hyperplasia, dysplasia and carcinoma, which is quite similar to that of tumors that develop in oral cancer patients (6). DMBA induced hamster buccal pouch carcinogenesis is therefore used as an ideal model for studying chemoprevention and oral cancer (7). The evaluated results were reported in hamster buccal pouch carcinogenesis may assist the clinicians in the treatment of oral cancer patients (8).

Glycoproteins are conjugated proteins in which carbohydrates are joined together covalently to asparagine or serine or threonine residue of polypeptide (9). The main sugar moieties in glycoproteins are glucose, galactose, fucose, mannose, and sialic acid as well as acetylated derivatives of hexosamine (10). Glycoconjugates are an important constituent of cell membrane and plays an essential role in cell function and cell membrane functions (11). It has been reported that elevated glycoconjugates are released into the circulation through increased turnover, secreation and /or shedding from malignant cells (12). Cytokeratin (CK) are the epithelial specific intermediate filament proteins, it over expression is closely linked to the epithelial tumors of stratified squamous cell origin (13). It is an sensitive marker in numerous molecular, biological, clinical and pathological studies have increased the knowledge on the alteration of cytokeratin expression in tumor biology (14). Intermediary filaments (IFS) are essential for intracellular components, underlying or reflecting distinct cellular properties and differentiation stages in epithelial organs (15). The expression of cytokeratin seems to be correlated with the malignant transformation in oral epithelium (16).

Chemoprevention is a novel and promising approach to control, inhibit or suppress the tumor formation by using natural or synthetic entities. Natural or synthetic agents that posses antimutagenic, anticarcinogenic, antioxidant, anti-lipid peroxidative and anti-cell proliferating properties are considered as good chemopreventive agents (17). A large number of phytochemical ingested in human diet exhibit anticarcinogenic and antimutagenic effects (18). Thymoquinone (TQ) is a bio-

*Corresponding author. Tel: 91-4144-239141 (Extn. *209)(Off.); Fax: 91-4144-239808; E-mail: suraj_cks@yahoo.co.in
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active compound obtained from volatile oil of Nigella sativa. The volatile oil of Nigella sativa was shown to contain about 24% thymoquinone (19). TQ is a pharmacologically active quinone. Several studies were documented that TQ possess several medicinal properties including analgesic, anti-inflammatory, protective effect on lipid peroxidation and oxidative damage, anticonvulsant and also antioxidant effect (20, 21). TQ may prove to be effective in treating prostate cancer, particularly in hormone refractory cases. TQ inhibits progression of prostate cancer cells from G1 to S phase (22, 23). TQ has been shown to induce apoptosis by p53-independent and p53 dependent pathways (24, 25). Growth inhibition of TQ is associated with inhibition of DNA synthesis and induction of cell cycle arrest (26).

However, no scientific reports were available on the literature for its membrane stabilizing effects of thymoquinone on cell surface glycoconjugates and cytokeratin expression against DMBA induced hamster buccal pouch carcinogenesis. The present study was therefore designed to examine the membrane stabilizing effects of thymoquinone on cell surface glycoconjugates and cytokeratin expression against DMBA induced hamster buccal pouch carcinogenesis.

RESULTS

Fig. 1 shows the histological features observed in the buccal mucosa of control and experimental animals in each group. We observed severe keratosis, hyperplasia and dysplasia and well differentiated tumor formation in hamsters painted with DMBA alone. Oral administration of TQ to DMBA treated hamsters significantly reduced the above said neoplasmic changes into mild conditions. Hamsters treated with thymoquinone alone showed no significant differences in histological features as compared to control hamsters.

Fig. 2 shows the cytokeratin expression observed in the buccal mucosa of control and experimental animals in each group. We observed expression of cytokeratin in hamster treated with DMBA alone as compared to control animals. Oral administration of TQ to DMBA treated hamsters significantly reduced the expression of cytokeratin in oral squamous cell carcinoma (OSCC). Hamsters treated with thymoquinone alone showed no significant differences in expression as compared to control hamsters.

Tables 1 and 2 show the status of glycoconjugates in plasma (protein bound hexose, protein bound hexosamine, total sialic acid, lipid bound sialic acid and fucose), erythrocyte membrane (protein bound hexose, protein bound hexosamine and total sialic acid) and buccal mucosa (protein bound hexose, total sialic acid, and fucose) of control and experimental animals in each group. The levels of glycoconjugates were significantly increased in plasma and buccal mucosa whereas decreased in erythrocyte membranes of tumor bearing hamsters as compared to control hamsters. Oral administration of TQ at a dose of 30 mg/kg b.wt. to DMBA painted hamsters brought back the status of glycoconjugates to near normal range in plasma, erythrocyte membrane and buccal mucosa. Hamsters treated with thymoquinone alone showed no significant differences in glycoconjugates status as compared to control hamsters.

DISCUSSION

Oral administration of TQ significantly prevented the incidence of oral neoplasm and cytokeratin expression in DMBA painted hamsters. This study indicates that TQ has potent che-
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Fig. 2. Cytokeratin expression observed in the buccal mucosa of control and experimental animals in each group. (A) Microphotograph showing low levels of cytokeratin expression in normal buccal mucosa tissue (×10), (B) Microphotograph showing over expression of cytokeratin in DMBA alone treated buccal mucosa tissue (×10), (C) Microphotograph showing moderate cytokeratin expression in DMBA + thymoquinone treated buccal mucosa tissue (×10), (D) Microphotograph showing low levels of cytokeratin expression in thymoquinone alone treated buccal mucosa tissue (×10).

Table 1. The levels of Protein bound hexose, hexosamine, sialic acid and fucose in plasma of control and experimental animals in each group (n=6)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Protein bound hexose (mg dl⁻¹)</th>
<th>Protein bound hexosamine (mg dl⁻¹)</th>
<th>Total sialic acid (mg dl⁻¹)</th>
<th>Lipid bound sialic acid (mg dl⁻¹)</th>
<th>Fucose (mg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td></td>
<td>86.54 ± 8.70³</td>
<td>74.2 ± 6.86³</td>
<td>47.15 ± 4.57⁷</td>
<td>11.75 ± 1.88⁷</td>
<td>7.38 ± 0.24⁴</td>
</tr>
<tr>
<td>2 DMBA</td>
<td></td>
<td>129.67 ± 9.23²</td>
<td>111.45 ± 7.46²</td>
<td>79.46 ± 9.49³</td>
<td>30.11 ± 2.76³</td>
<td>16.39 ± 1.43³</td>
</tr>
<tr>
<td>3 DMBA + Thymoquinone</td>
<td></td>
<td>106.19 ± 7.86⁶</td>
<td>90.54 ± 8.28⁶</td>
<td>58.43 ± 4.57⁷</td>
<td>19.35 ± 3.70⁷</td>
<td>11.44 ± 2.17⁷</td>
</tr>
<tr>
<td>4 Thymoquinone alone</td>
<td></td>
<td>87.25 ± 7.69⁰</td>
<td>75.51 ± 6.80⁰</td>
<td>48.03 ± 4.57⁷</td>
<td>12.47 ± 1.20⁰</td>
<td>8.18 ± 1.50⁰</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six hamsters in each group. aValues are not differ significantly at P < 0.05 (DMRT). b, cValues are differ significantly at P < 0.05 (DMRT).

Table 2. The levels of Protein bound hexose, hexosamine and total sialic acid in erythrocyte membrane and buccal mucosa of control and experimental animals in each group (n=6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DMBA</th>
<th>DMBA + Thymoquinone</th>
<th>Thymoquinone alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein bound hexose (mg dl⁻¹)</td>
<td>126.65 ± 10.55³</td>
<td>86.74 ± 8.03³</td>
<td>108.59 ± 7.85⁷</td>
<td>127.42 ± 11.44⁴</td>
</tr>
<tr>
<td>Protein bound Hexosamine (mg dl⁻¹)</td>
<td>126.65 ± 10.55³</td>
<td>86.74 ± 8.03³</td>
<td>108.59 ± 7.85⁷</td>
<td>127.42 ± 11.44⁴</td>
</tr>
<tr>
<td>Total sialic acid (mg dl⁻¹)</td>
<td>126.65 ± 10.55³</td>
<td>86.74 ± 8.03³</td>
<td>108.59 ± 7.85⁷</td>
<td>127.42 ± 11.44⁴</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein bound hexose (mg dl⁻¹)</td>
<td>106.11 ± 9.08⁶</td>
<td>150.93 ± 14.20⁸</td>
<td>122.49 ± 8.56⁷</td>
<td>108.04 ± 10.62⁴</td>
</tr>
<tr>
<td>Total sialic acid (mg dl⁻¹)</td>
<td>106.11 ± 9.08⁶</td>
<td>150.93 ± 14.20⁸</td>
<td>122.49 ± 8.56⁷</td>
<td>108.04 ± 10.62⁴</td>
</tr>
<tr>
<td>Fucose (mg dl⁻¹)</td>
<td>106.11 ± 9.08⁶</td>
<td>150.93 ± 14.20⁸</td>
<td>122.49 ± 8.56⁷</td>
<td>108.04 ± 10.62⁴</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six hamsters in each group. Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT). aValues are not differ significantly at P < 0.05 (DMRT). b, cValues are differ significantly at P < 0.05 (DMRT).

Screening of glycoconjugates and cytokeratin expression is helpful to assess the cancer in patients with malignant neoplasm (27). Glycoproteins play a significant role in contributing to the surface properties of the cells and also important role in tumorigenesis and as mediators of immunological specificity (28). The sugar moieties of glycoprotein have also been implicated in the transport of metabolites across the cell membranes (29). The malignant transformation is usually associated with molecular changes such glycosylation of glycoprotein’s and glycolipids (30). It is an essential for cell-cell communication, cell differentiation, intercellular recognition, and as re-
ceptors for many hormones and viruses (31). The measurement of serum biochemical markers such as hexose, hexosamine and sialic acid in oral pre-cancerous and cancerous lesions may be useful in the diagnosis of patients with oral pre-cancer or cancer (32). It also observed a direct relationship between glycoproteins and tumorigenesis (33). Aberrant glycosylation of cell surface glycoproteins has been observed for tumor cells and its involvement in the metastatic processes (34).

The human body stimulates the synthesis of glycoproteins in the liver associated with malignant transformations, which subsequently enter into the circulation (35). The decreased levels of erythrocyte membrane glycoprotein may be due to the increased membrane degradation (36). Elevated levels of plasma glycoprotein concentration in tumor bearing animals can therefore be related to an increased synthesis in liver or tumor tissues itself with subsequent shedding into plasma (37). Previous studies in our laboratory reported that malignant cells have more sialic acid in their cell membrane than in normal cells. Several studies reported that marked elevation of total sialic acid and lipid bound sialic acid in serum were found to reflect tumor burden and correlated well with stages of cancer (38, 39).

Human body requires fucose as one of the essential sugar for optimal function of cell-cell communication. Fucose plays a significant role in many diseases including cancer and its spread (40). Increase in fucose content in tumor tissue and plasma is probably due to increased turnover of malignant cells with subsequent shedding into circulation (41). An increase glycoprotein's levels in urine were also reported with oral cancer patients reflects elevation of sialyl transferase activity in tumor tissue (42). Suresh et al., reported that increased sialyl transferase may be responsible for increased expression of cell surface glycoconjugates during neoplastic transformation (43). Our results corroborate these observations.

A variety of biological markers mainly involved in cell proliferation and apoptosis have been described. In various other entities it could also be shown that the aberrant expression of cytokeratins as a main family of intermediate filaments might add additional prognostic significance (44). Several studies reported that associations between changes in the intermediate filament expression and altered cellular behaviour significant changes in the pattern and distribution of cytokeratins have been observed in a wide range of epithelial carcinomas (45).

Oral administration of TQ to DMBA painted hamsters significantly normalized and inhibited the abnormalities seen in cell surface glycoconjugates in the tumor tissues and circulation during carcinogenesis, and restored the expression of cytokeratin, which indicates their membrane stabilizing effect of TQ during neoplastic transformation. The protective role of TQ on cell surface glycoconjugates is probably due to their inhibitory role on glycoprotein synthesis or on the activity of the glycosyl transferase. So, further studies are warranted to elucidate the mechanistic pathway on effect of TQ in DMBA induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Chemicals

The carcinogen, 7,12-dimethylbenz(a)anthracene and Thymoquinone was obtained from Sigma-Aldrich Chemical Pvt. Ltd, Bangalore, India. All other chemicals used were of analytical grade, marketed by Himedia laboratories, Bangalore and Sisco Research Laboratories Pvt Ltd, Mumbai, India.

Animals

Male golden Syrian hamsters, 8-10 weeks old, weighing 80-120 g were purchased from National Institute of Nutrition, Hyderabad, India and maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided standard pellet diet and water ad libitum. The animals were maintained under controlled conditions of temperature and humidity with a 12h light /dark cycle.

Experimental protocol

The local institutional animal ethics committee, Annamalai University, Annamalai Nagar, India, has approved the experimental design. A total number of 24 male golden Syrian hamsters were randomized into 4 groups of 6 animals in each. Group I animals were served as untreated control. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin three times per week for 14 weeks on the left buccal pouches (No: 4 brush). Group II animals received no other treatment. Group III animals were orally administered with Thymoquinone (30 mg kg⁻¹ b.wt) starting one week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the scarification of the animals. Group IV animals received TQ alone throughout the experimental period. The experiment was terminated at the end of 14th week and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on plasma, erythrocytes and buccal mucosa of control and experimental animals in each group.

Biochemical analysis

After plasma separation, the erythrocyte membrane was prepared by the method of Dodge et al., (46). Modified by Quist (47). The protein bound hexose, hexosamine, total sialic acid and fucose in plasma, erythrocyte membrane and buccal mucosa tissues were estimated by the methods of Niebes et al., (48), Wanger (49), Warren (50) and Dische and Shettles (51) respectively. Plasma lipid bound sialic acid level was determined by the method of katopodis and Stock (52).

Immunohistochemical analysis

Immunohistochemistry was performed on 4-μm thick (TMA) tissue microarray sections. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 min
in methanol containing 0.3% hydrogen peroxide. After antigen retrieval a cooling off period of 20 min preceded the incubation of the primary antibody. Antibodies were detected by a standard avidin-biotin complex method with a biotinylated rabbit anti-mouse antibody (Dako) and an avidin-biotin complex (Dako). The staining was developed with diaminobenzidine before the slides were mounted; all sections were counterstained for 45 sec with hematoxylin and dehydrated in alcohol and xylene (53). Appropriate negative controls and positive controls were used throughout the expression of cytokeratin was determined by pathologist.

Statistical analysis
Values are expressed as mean ± SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT). The values were considered statistically significant if the P value were less than 0.05.

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REFERENCES