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MODULATING EFFECT OF GLYCYRRHIZIN ON PLASMA GLYCOCONJUGATES STATUS IN 7, 12- DIMETHYL BENZ (A) ANTHRACENE INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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Abstract

Oral cancer starts in the squamous cells that cover the surfaces of the mouth, tongue, and lips. Cancer of the oral cavity has multifactorial aetiologies and is frequently associated with chewing of betel quid containing tobacco, in addition to smoking and alcohol consumption. Glycoproteins are complex proteins in which both a protein and a carbohydrate are joined together in the covalent linkage. Many functional proteins are released by cells into the blood circulation as glycoproteins. DMBA a potent procarcinogen has been used to develop Oral tumors in the buccal mucosa of golden Syrian hamsters. The levels of plasma glycoconjugates were analyzed using standard colorimetric methods. Glycyrrhizin (45 mg//kg b.w) significantly prevented the tumor formation, tumor volume, and burden in DMBA painted hamsters, which indicates its potent chemopreventive efficacy in experimental oral carcinogenesis. Glycyrrhizin administration to DMBA treated hamsters significantly reduced the levels of glycoconjugates to near normal range. The present study thus explores the modulating effect of Glycyrrhizin in DMBA induced oral cancer.

***Keywords*:** Glycyrrhizin, glycoproteins, hexose, hexosamine, sialic acid, fucose.

Introduction

Oral cancer proceeds from normal to dysplastic lesions and then to squamous cell carcinoma. It usually starts in the squamous cells that cover the surfaces of the mouth, tongue, and lips. Cancer of the oral cavity has multifactorial aetiologies and is frequently associated with chewing of betel quid containing tobacco, in addition to smoking and alcohol consumption (Petti, 2005; Scully, 2005). The incidence and mortality rate of oral cancer vary widely across the world. The higher incidences of oral cancer are reported every year from developing countries, particularly from India. Oral squamous cell carcinoma is predominantly a disease of the human population in the fifth to eighth decades of lift (Moor, *et al.*, 2000). Oral cancer accounts for 40-50% of all cancers in India, where approximately 14 deaths per hour are reported to occur due to this form of cancer (Petersen, 2009).

The carcinogen, 7,12–dimethylbenz(a)anthracene (DMBA), can initiate and promote the development of oral carcinoma of the buccal mucosa through distinct premalignant lesions. DMBA- induced experimental oral cancer is the most widely- accepted experimental model for studying chemoprevention of oral cancer, since it has several morphological and histological similarities with human oral carcinoma (Miyata *et al.*, 2001; Shklar, 1999) DMBA induced oral carcinogenesis is, therefore, an ideal model to study the chemopreventive effect of natural and synthetic agents (Sporn and Liby, 2005).

Glycoproteins are complex proteins in which both a protein and a carbohydrate are joined together in the covalent linkage. Glycoproteins are proteins with one or more heterosaccharide chains that contain hexose, hexosamine, sialic acid and fucose as predominantly sugar moieties. Many functional proteins are released by cells to the blood circulation as glycoproteins. The cell membrane contains between 2% and 10% carbohydrates in the form of glycolipids and glycoproteins (Neufeld and Ashwell, 1987). Glycoproteins are important components of the intercellular matrix, cell membrane, and membrane of the subcellular organelles.

Biologically important substances such as enzymes, hormones, and antibodies represent these glycoproteins. Neoplastic transformation of a variety of cell types is associated with changes in the composition of membrane glycoproteins (Dwivedi *et al.*, 1990). Profound reports suggest that glycoconjugates levels were markedly altered during neoplastic transformations (Manoharan *et al.*, 2008). Sialic acid can potentially inhibit intercellular interaction under their negative charge. Serum sialic acid levels have been used as laboratory markers in cancers (Goodarzi *et al.*, 2005). Fucose plays an important role in cell-cell communication. Abnormal levels of fucose in the cell surface may lead to malignant transformation and metastasis. Serum fucose, a terminal pentose sugar of the glycoprotein chains, is detectable in the blood of both normal persons and persons with different types of malignancies. Total sialic acid and lipid-bound sialic acid elevation in serum and tumor tissues are probably related to the increased turnover of malignant cells (Rao *et al.*, 1998).

Licorice root, a traditional herbal remedy, has been used for the treatment of several disorders including cancer (Rahman *et al.*, 2007). Glycyrrhizin, a triterpenoid saponin, is well known for its anti-inflammatory potential in Chinese medicine (Jancinova *et al.*, 2007). Glycyrrhizin is the major component of Glycyrrhiza glabra root, with a concentration of 1-9%. Glycyrrhizin is a glycosylated saponin, containing one molecule of glycyrrhizic acid, with structural similarities to hydrocortisone, and two molecules of glucuronic acid. It has been attributed to numerous pharmacologic effects like anti-inflammatory, anti-viral, anti-tumor and hepatoprotective activities. Glycyrrhizin has been used clinically in Japan for patients with active chronic hepatitis.

The present study has evaluated the modulating effect of Glycyrrhizin on plasma glycoconjugates in DMBA induced oral carcinogenesis.

Materials and Methods

a) Chemicals

DMBA and Glycyrrhizin were obtained from Sigma–Aldrich Chemicals Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade and were purchased from HiMedia Laboratories, Mumbai, India.

b) Samples used in the Present Study

Blood samples: Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 1000g for 15 minutes.

c) Experimental Design

Describe the experimental protocols. Group, I hamsters served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Groups II hamsters received no other treatment.

Group III hamsters were orally given Glycyrrhizin at a dose of 45 mg/kg b.w /day, starting 1 week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Group IV hamsters received oral administration of Glycyrrhizin alone throughout the experimental period. The experiment was terminated at the end of 16 weeks and all animals were sacrificed by cervical dislocation.

Estimation of Glycoconjugates Profile

The precipitate obtained after treating the plasma with 95% ethanol was used for the estimation of protein-bound hexose, hexosamine, sialic acid, and fucose. The defatted buccal mucosa tissues obtained after treating the plasma with methanol and chloroform were used for the estimation of glycoproteins. To the plasma, remaining after lipid extraction, 0.1N H_2SO_4 was added and hydrolyzed at 80˚C for 1hour. It was cooled and the aliquot was used for sialic acid estimation. To the remaining solution, 0.1N NaOH was added and kept in an ice bath for 1 hour. From these aliquots, protein-bound hexose, hexosamine and fucose were estimated.

Estimation of Protein Bound Hexose

The protein-bound hexose in the plasma was estimated by the method of (Niebes., 1972). To the glycoprotein extract, orcinol and sulphuric reagent were added and heated for 15 minutes at 80˚C. 0.5 ml of aliquot was mixed with 0.5 ml 5% phenol and 2.5 ml concentrated sulphuric acid was added. 0.5 ml of 0.1 N NaOH for blank and standards in the concentration range of 40-200 µg were treated similarly. The tubes were then heated in a boiling water bath for 20 minutes and the absorbance of the color developed was measured at 540nm. The concentration of protein-bound hexose was expressed as mg/dl for plasma.

Estimation of Protein Bound Hexosamine

The protein-bound hexosamine in the plasma was estimated by the method (Wagner, 1979). 0.1 ml of extract was treated with 2.5 ml of 3N HCl for 6 hours in a boiling water bath and then neutralized with 6N NaOH. To 0.8 ml of neutralized sample, added 0.6 ml of acetylacetone reagent. The tubes were heated in a boiling water bath for 30 minutes. After cooling 2 ml of Ehrlich's reagent was added and mixed well. Blank contained 1.0 ml NaOH was treated in the same way. The color developed was read at 540 nm. The concentration of protein-bound hexosamine was expressed as mg/dl for plasma.

Estimation of Sialic Acid

The total sialic acid in the plasma was estimated by the method (Warren, 1959). To 0.5 ml of sample (plasma) in a test tube, 0.5 ml of water and 0.25 ml of periodic acid were added and incubated at 37˚C for 30 minutes. To this 0.25 ml of sodium m-arsenate and 2 ml of TBA were added and heated in a boiling water bath for 6 minutes. It was cooled and 5 ml of acidified butanol was added. The absorbance by the extract in the organic layer was read at 640 nm against the reagent blank. The concentration of total sialic acid was expressed as mg/dl for plasma.

Estimation of Fucose

Fucose in the plasma was estimated by the method of (Dische and Shettles, 1948). Plasma was treated with H_2SO_4 for 10 minutes followed by the addition of cysteine hydrochloride. To 0.5 ml of aliquot, 4.5 ml of the sulphuric acid-water mixture was added. The tubes were kept in a boiling water bath for 3 minutes and cooled. 0.1ml of cysteine hydrochloride reagent and 0.5 ml of 0.1 N NaOH were added. Blank and standards in the concentration range of 5-25 µg were also treated the same way. After 75 minutes in dark, the absorbance of the color developed was noted at 620nm.

Statistical Analysis

The values are expressed as mean ± SD. The statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), using SPSS version 12.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values are considered statistically significant if the *p-value* was less than 0.05.

Results and Discussion

The levels of glycoconjugates in plasma protein-bound hexose, hexosamine, sialic acid and fucose of control and experimental hamsters in each group are shown in (figures 1 – 4) respectively. The levels of glycoconjugates in plasma were significantly increased in hamsters painted with DMBA as compared to control hamsters. Oral administration of Glycyrrhizin (45 mg/kg b.w) to DMBA-painted hamsters brought back the levels of the above-mentioned glycoconjugates to near-normal ranges. No significant difference was noticed in the levels of plasma glycoconjugates between Glycyrrhizin–alone treated hamsters and control hamsters.

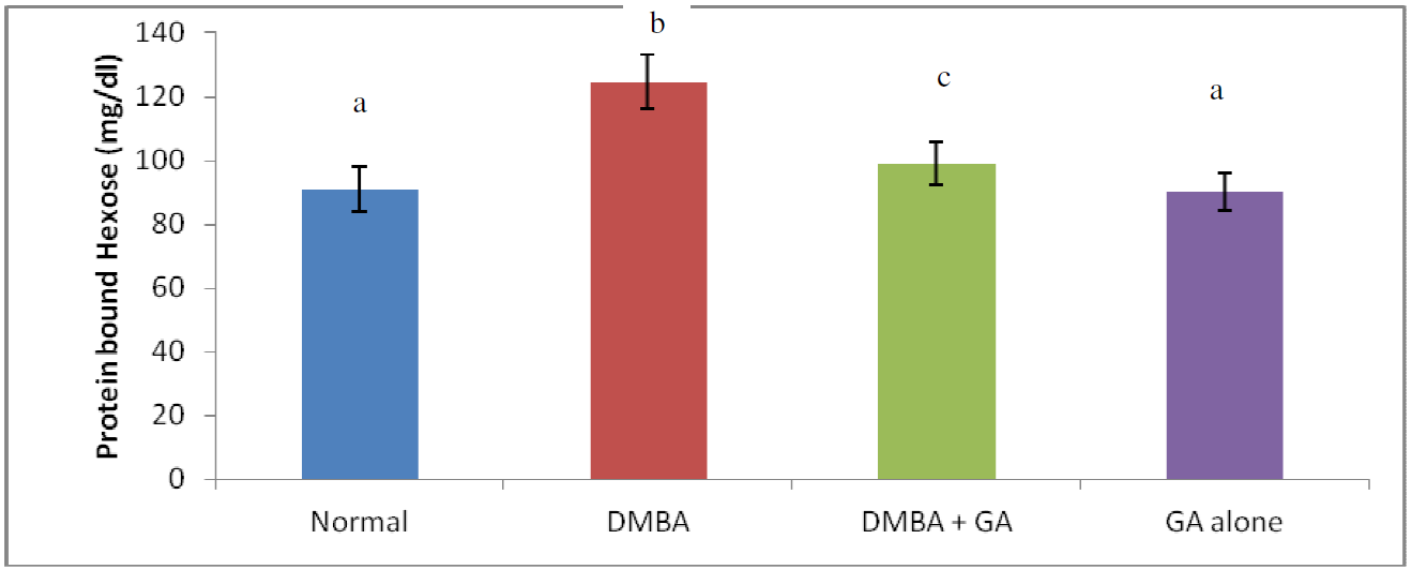

Fig. 1 : Status of protein bound hexose in control and experimental hamsters in each group

The values are expressed as mean ± SD for 10 hamsters in each group. Values that are not sharing a common superscript later between-group differ significantly at 'p' less than 0.05.

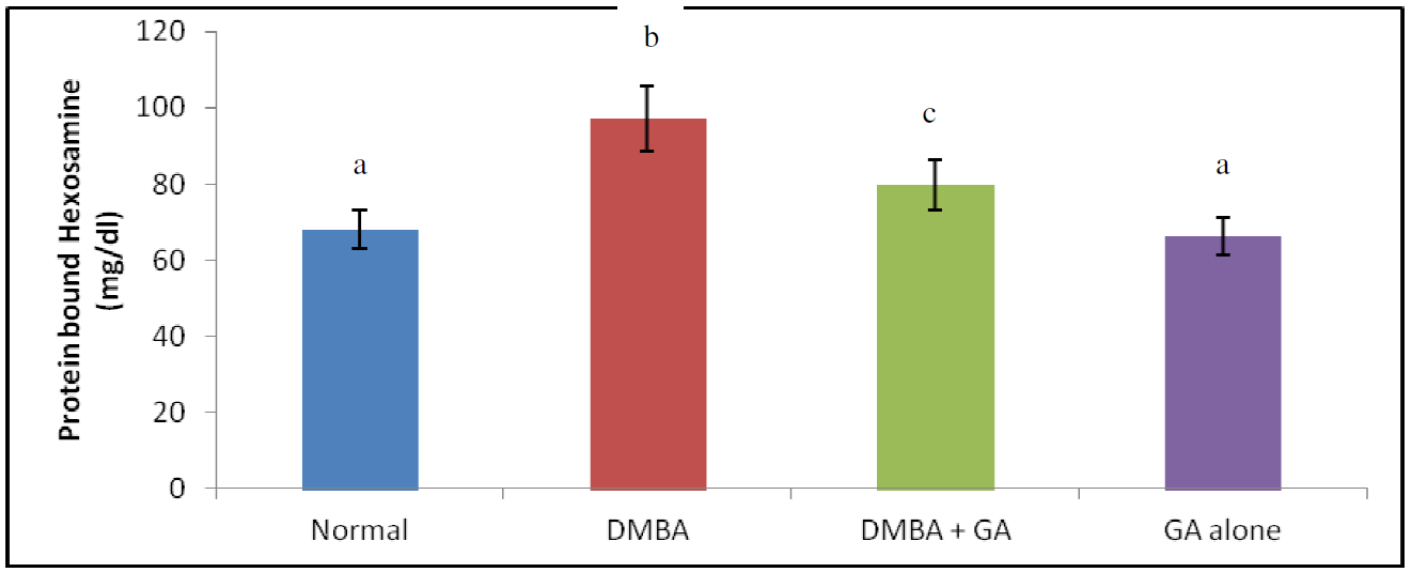

Fig. 2: Status of protein bound hexosamine in control and experimental hamsters in each group

The values are expressed as mean ± SD for 10 hamsters in each group. Values that are not sharing a common superscript later between-group differ significantly at 'p' less than 0.05.

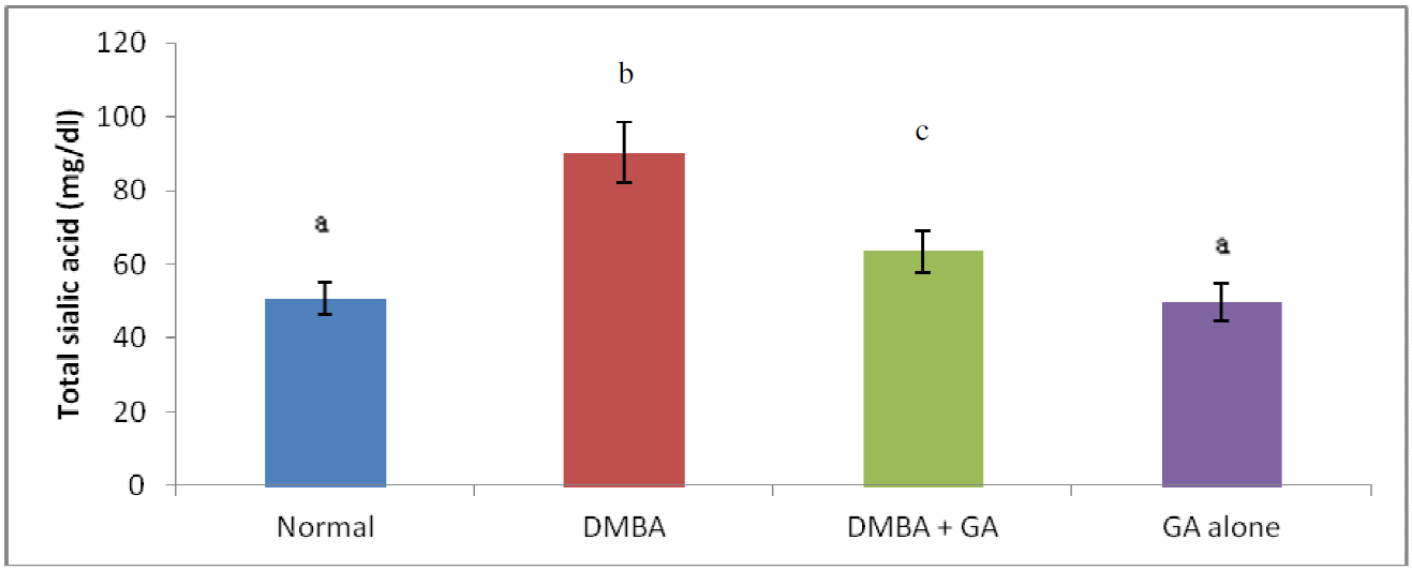

Fig. 3: Status of total sialic acid in control and experimental hamsters in each group

The values are expressed as mean ± SD for 10 hamsters in each group. Values that are not sharing a common superscript later between-group differ significantly at 'p' less than 0.05.

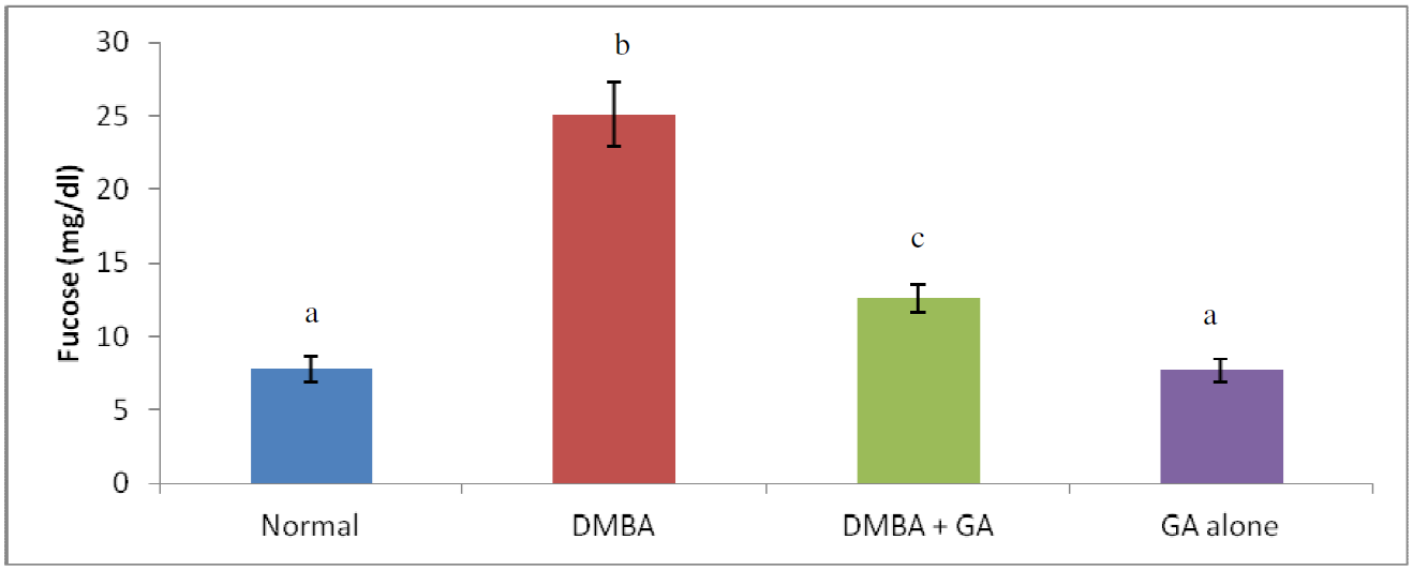

Fig. 4 : Status of fucose in control and experimental hamsters in each group

The values are expressed as mean ± SD for 10 hamsters in each group. Values that are not sharing a common superscript later between-group differ significantly at 'p' less than 0.05.

Glycoproteins and glycolipids are major constituents of the cell membrane, and the levels of cell surface glycoconjugates are thus important in malignancy. Measurement of glycoproteins along with other clinical and biochemical variables could help in the disease diagnosis, staging of disease, and assessing therapeutic response. The status of protein-bound hexose, hexosamine, sialic acid, and fucose are used as biomarkers in carcinogenesis. We have observed elevated levels of glycoconjugates during oral malignant transformation. Elevated levels of serum sialic acid in precancerous and cancerous conditions of the oral cavity have been observed (Goodarzi *et al.*, 2005). Elevated levels of protein-bound hexose and hexosamine, fucose, and sialic acid have been found in various malignant conditions (Manoharan *et al.*, 2012). Abnormal levels of plasma and tumor tissue glycoconjugates were reported in several experimental carcinogeneses. Increased levels of glycoconjugates could be due to abnormal expression or synthesis of glycoproteins in the tumor tissues with subsequent shedding into plasma.

Conclusion

In the present study, oral administration of Glycyrrhizin (45 mg//kg b.w) significantly prevented the tumor formation, tumor volume and burden in DMBA painted hamsters, which indicates its potent chemopreventive efficacy in experimental oral carcinogenesis Glycyrrhizin not only prevented the cancer formation but also inhibited the abnormal synthesis of cell surface glycoconjugates as evidenced by decreased levels of plasma glycoconjugates in DMBA + Glycyrrhizin treated hamsters. The modulating effect of Glycyrrhizin on cell surface glycoconjugates could probably be due to its inhibitory role in glycoprotein synthesis or on the activity of the glycosyltransferases. The present study thus demonstrates the protective efficacy of Glycyrrhizin on abnormal glycoconjugates expression during DMBA induced oral carcinogenesis.

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