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ISSN: 0975-7538
Research Article

Carnosic acid protects cell surface glycoconjugates abnormalities during 7,12-dimethylbenz(a)anthracene(DMBA) induced oral carcinogenesis

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ABSTRACT

Aim of the present study was to focus the protective effect of carnosic acid on 7,12-dimethylbenz(a)anthracene (DMBA) induced cell surface glycoconjugates (Protein bound hexose, Hexosamine, Lipid bound sialic acid, Total sialic acid and Fucose) abnormalities in the plasma and buccal mucosa of golden Syrian hamsters. Oral squamous cell carcinoma was developed in the buccal pouch of hamsters by painting with 0.5% DMBA three times a week for 14 weeks. Glycoconjugates status was assessed both histologically and biochemically in the buccal mucosa of DMBA treated hamsters. The levels of glycoconjugates were increased in both plasma and buccal mucosa of DMBA treated hamsters. Oral administration of carnosic acid at a dose of 10mg/kg bw brought back the status of glycoconjugates to near normal concentrations in DMBA treated hamsters. Present results thus suggest that carnosic acid protected DMBA induced cell surface abnormalities during DMBA induced hamster buccal pouch carcinogenesis.

Keywords: Oral cancer; Glycoconjugates; Carnosic acid.

INTRODUCTION

Oral squamous cell carcinoma, one of the most common malignant neoplasm worldwide, is predominantly a disease of human population in the fifth to eighth decades of life. Oral carcinogenesis is preceded by a sequence of precancerous lesions such as leukoplakia, erythroplakia and oral submucous fibrosis. Higher prevalence of oral cancer is frequently associated with chewing of betel quid with and without tobacco smoking and alcohol consumption (Johnson 2001). 7,12-dimethylbenz(a)anthracene(DMBA) is one of the most common chemical carcinogen employed to induce oral carcinogenesis in the buccal pouch of golden Syrian hamsters (Manoharan, et al., 2010). The precancerous and cancerous lesions induced by this potent site and organ specific carcinogen not only resemble human oral precancerous and cancerous lesions, but also expresses similar biochemical and molecular markers that are expressed in human oral carcinoma (Miyata, et al., 2001).

Glycoproteins and glycolipids are important constituents of cell membrane. Cellular glycosylation changes are associated with diverse types of neoplastic transformation (Dabelsteen 1996). Altered levels of glycoproteins and glycolipids perform crucial role in aberrant cell-cell recognition, cell adhesion, antigenicity and invasiveness (Varki and Schauer 2008). Sialic acids, a nine-carbon keto sugars, is involved in maintaining the ionic balance, regulation of the immune response, the progression and spread of human malignancies and in certain aspects of human evolution (Schauer 2009). Cell surface sialic acids also play an important role in cell – cell communication. Recent studies showed that the levels of total sialic acid and lipid bound sialic acid were higher in patients with cancer than normal subjects (Chen and Varki, 2010). Fucose (6-deoxy-L-galactose), a monosaccharide that is found on glycoproteins and glycolipids, is essential for optimal function of cell to cell communication. An altered level of fucose plays a significant role in many diseases, including cancer and its spread. Altered fucosylation of the cell surface was contributed to the ability to escape from immune control. Higher levels of fucose have been reported in patients with oral cancer and precancerous lesions (MacDougall, et al., 1987).

Carnosic acid, the primary phenolic diterpene, has been received considerable attention due to its diverse pharmacological and biological actions (Wijeratne, et al., 2007). It is abundantly present in the leaves of rosemary (*Rosemarinus officinalis).* Experimental stud-

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Received on: 21-02-2011
Revised on: 14-03-2011
Accepted on: 17-03-2011

ies demonstrated anti-proliferative, anti-inflammatory, anti-tumorigenic and neuro-protective effects of carnosic acid (Cheung, et al., 2007). Carnosic acid induced cell cycle arrest predominantly at G2/M phase (Visanji, et al.,2006). Carnosic acid decreased metabolic activation and increased detoxification of benz(a)pyrene, identifying it as promising candidate for chemopreventive programs (Peng, et al.,2007)**.** Previous studies from our laboratory have demonstrated the antigenotoxic and chemopreventive potential of carnosic acid in DMBA treated hamsters (Manoharan, et al., 2010). The present study focused the protective effect of carnosic acid on cell surface glycoconjugates abnormalities during DMBA induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Chemicals

DMBA and carnosic acid were obtained from Sigma–Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, purchased from Hi-media Laboratories, Mumbai, India.

Animals

Male golden Syrian hamsters, aged 8–10 weeks, weighing 80–120 g, were purchased from the National Institute of Nutrition, Hyderabad, India and were maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed five in a polypropylene cage and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water *ad libitum.* The animals were maintained under controlled conditions of temperature (27±2°C) and humidity (55±5%) with a 12h light/dark cycle.

EXPERIMENTAL DESIGN

The Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design. A total number of 40 hamsters were randomized into four groups and each group contained 10 hamsters. Group I animals served as the control and were treated with liquid paraffin (vehicle) alone three times a week for 14 weeks on their left buccal pouches. Group II animals were treated with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II animals received no other treatment. Group III animals were treated with DMBA as in group II, received in addition oral administration of carnosic acid (10 mg/kg body weight/day), dissolved in 1 ml of 5 % dimethyl sulphoxide (DMSO), starting 1 week before exposure to the carcinogen and continued on alternate days to DMBA painting until the animals were sacrificed. Group IV animals received oral administration of carnosic acid (10 mg/kg body weight/day) alone, as in group III, throughout the experimental period. The experiment was terminated at the end of 16 weeks and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on the plasma, liver and buccal mucosa tissues. For histopathological examination, buccal mucosa tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2–3 µ m sections were cut in a rotary microtome and stained with haematoxylin and eosin. For detection of glycoconjugates, the tissue sections of buccal mucosa 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.

BIOCHEMICAL STUDIES

The precipitate obtained after treating the plasma with 95 % ethanol was used for the estimation of protein bound hexose and hexosamine. The defatted tissues obtained after treating buccal mucosa with methanol and chloroform was used for the estimation of glycoprotein. To the dry defatted tissues remaining after lipid extraction, 0.1N H_2SO_4 was added and hydrolyzed at 80°C for 1h. It was cooled and the aliquot was used for sialic acid estimation. To the remaining solution, 0.1N sodium hydroxide was added and kept in an ice bath for 1 h. From these aliquots, protein bound hexose and fucose were estimated. The protein bound hexose, hexosamine, total sialic acid and fucose were estimated by the methods of Niebes (Niebes, et al., 1972), Wagner (Wagner, et al., 1979), Warren (Warren, et al., 1959) and Dische and Shettles (Dische and Shettles, 1948) respectively. Plasma lipid bound sialic acid level was determined by the method of Katopodis and Stock (Katopodis and Stock, 1980).

Statistical analysis

The data 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 results were considered statistically significant if the p values were 0.05 or less.

RESULTS

Figure 1(a-e) shows the levels of glycoconjugates in plasma (protein bound hexose, hexosamine, total sialic acid, Lipid bound sialic acid and fucose) and Figure 2(a-c) shows the buccal mucosa glycoconjugates (protein bound hexose, total sialic acid and fucose) of control and experimental hamsters in each group. The levels of glycoconjugates in plasma and buccal mucosa were significantly increased in DMBA alone painted hamsters (group 2) as compared to control hamsters. Oral administration of carnosic acid to DMBA painted hamsters (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 buccal mucosa glycoconjugates in carnosic acid alone

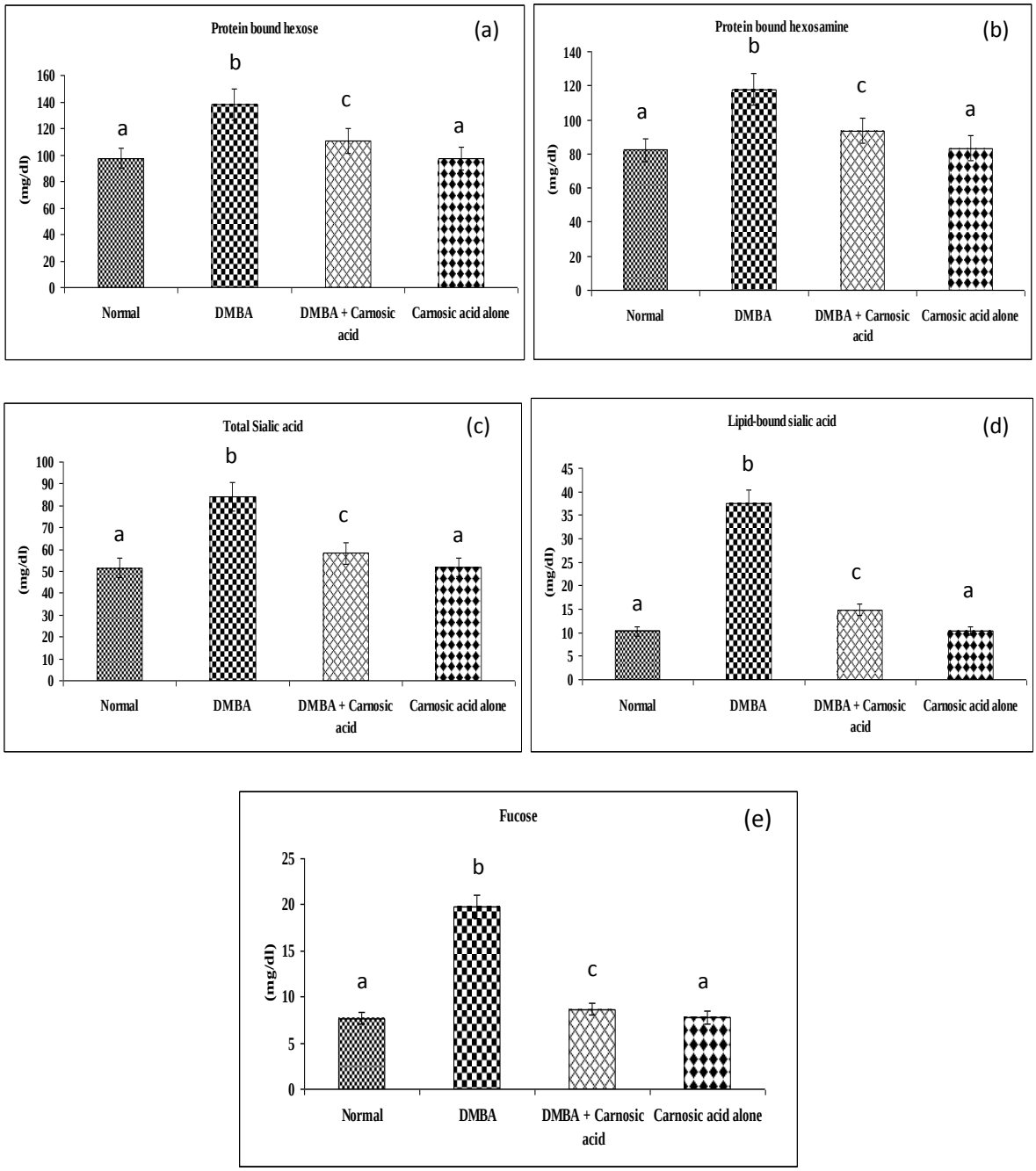

Figure 1: (a-e) shows the levels of glycoconjugates in plasma (protein bound hexose, hexosamine, total sialic acid, lipid bound sialic acid and fucose) of control and experimental hamsters in each groups

Values are expressed as mean± SD (n=10). Values that are not sharing a common superscript differ significantly at $p<0.05$.

(group 4) treated hamsters as compared to control hamsters (group 1).

Figure 3 (a - d) shows glycoconjugates expression pattern in the buccal mucosa of control and experimental animals in each group. The glycoconjugates expression pattern was analysed as evidenced by periodic acid Schiff's staining in the buccal mucosa. We observed increased glycoconjugates expression in the buccal mucosa of tumor bearing hamsters (Group 2; figure. 3b). Oral administration of carnosic acid to DMBA painted hamsters significantly reduced the expression of glycoconjugates in the buccal mucosa (Group 3; figure 3c). Glycoconjugates expression pattern was normal in carnosic acid alone treated (Group 4; figure 3d) and control (Group 1; figure 3a) hamsters.

DISCUSSION

In recent years, considerel effects have been focused to emphasize the significance of cell- surface glycoconjugates during malignant transformation. Neoplastic transformation is often associated with profound alterations in cell membrane glycosylation. Profound studies documented that glycoconjugates levels could be

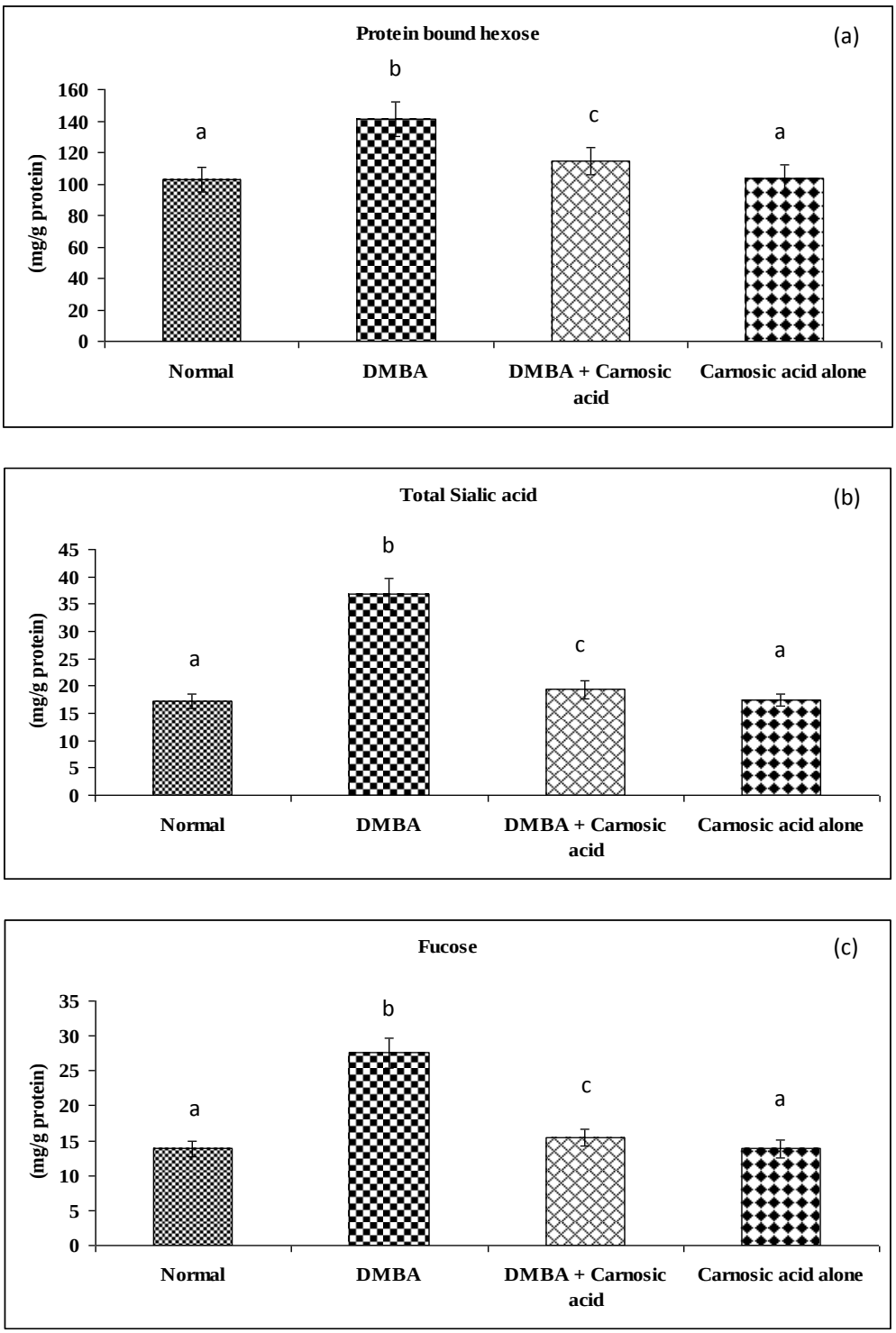

Figure 2: (a-c) shows the levels of glycoconjugates in buccal mucosa (protein bound hexose, total sialic acid and fucose) of control and experimental hamsters in each group

Values are expressed as mean± SD (n=10). Values that are not sharing a common superscript differ significantly at $p<0.05$

a promising approach for the early detection, diagnosis and prognosis of several cancers (Rajalingam, et al., 2008). Evaluation of serum or plasma glycoconjugates could help in the diagnosis of patients with oral pre-cancer or cancer (Manoharan, et al., 2004). Atypical glycosylation of cell surface carbohydrates and reduction in epithelial cell surface carbohydrates is associated with malignant transformation (Dabelsteen 1996). Glycoconjugates are secreted from membrane into extracellular fluid in cancerous conditions. A loss in epithelial cell surface carbohydrate during experimental oral carcinogenesis has been reported (Thirunavukarasu, et al., 2003). Profound studies documented that the synthesis of glycoproteins increased in tumour

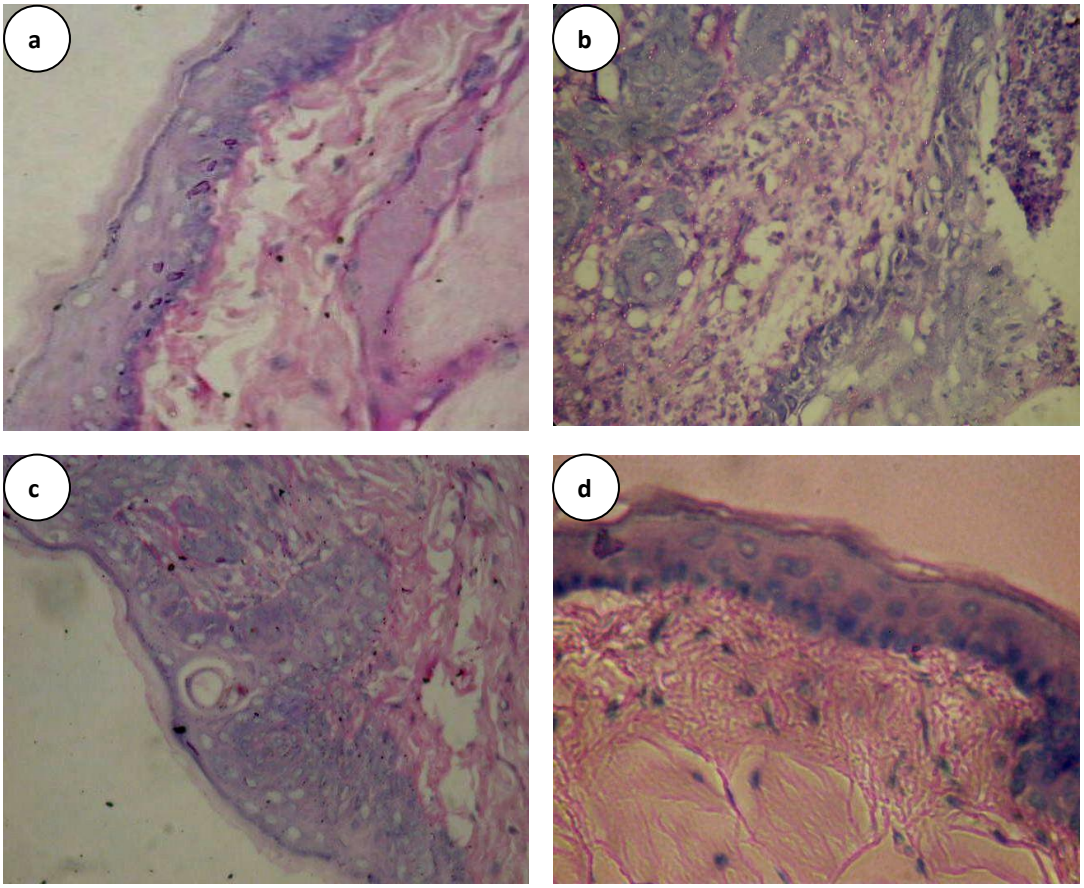

Figure 3: (a - d) shows glycoconjugates expression pattern in the buccal mucosa of control and experimental animals in each group

(a) Normal glycoconjugates expression in the control hamsters (40X)

(b) Overexpression of glycoconjugates in hamsters treated with DMBA alone (40X)

(c) Lowered expression of glycoconjugates in DMBA + carnosic acid treated hamsters (40X)

(d) Normal glycoconjugates expression in hamsters treated with carnosic acid alone (40X)

cells (Manoharan, et al., 2004). Highly proliferated cells were often found to have higher concentrations of total glycoproteins (Manoharan, et al., 2008). Increased levels of plasma glycoproteins in tumour bearing hamsters could be due to spontaneous release of glycoproteins from tumour cells or due to increased synthesis in tumour cells with subsequent shedding into plasma.

Sialic acids, the end moieties of the carbohydrate chain of glycoconjugates, are reported to be elevated during malignancy (Senthil, et al., 2007). Tumor cells have twice the concentration of sialic acid as compared to normal cells (Manoharan, et al., 2004). Elevated levels of sialic acid in tumor tissues is due to selective increase in existing specific sialylated sequence or tumor associated denovo synthesis of specific sialylated sequence (Goodarzi, et,al., 2005). Several studies reported elevated serum total sialic acid (TSA), lipid bound sialic acid (LSA) and TSA to total protein ratio, in various malignancies (Pugalendhi, et al., 2011). A positive correlation between lipid bound sialic acid levels and tumour, staging, degree of metastasis and recurrence of diseases has been reported (Vasanti, et., 1998). Studies have shown that malignant changes are accompanied by increased expression of membrane associated fucose containing macromolecules (Kim, et al., 1982; Rao, et al., 1998). Altered metabolism of a L-fucose play an important role in the malignant behaviour of tumour cells, which include underexpression, overexpression or neoexpression of fucose-containing carbohydrate structures of specific N-glycans, mucin-type O-glycans and glycoproteins of the cell surface as well as elevated serum fucose concentrations (Wang, et al., 1995). Tumor itself may contribute to circulating fucose either due to spontaneous release of glycoproteins as the mass grows or as a result of host cell damage through carcinogen treatment (Glick, et al., 1978). Sialic acid and fucose, important constituents of glycoproteins, are released into the circulation through increased turnover, secretion, and/or shedding from the malignant cells (Narayanan, et al., 1994). Our results are line with these findings. The present study also confirmed the accumulation or increased expression of glycoconjugates in the tumour cell surface with

histopathological studies using Periodic acid Schiff's (PAS) staining.

Oral administration of carnosic acid at a dose of 10mg/kg bw to DMBA treated hamsters brought back the status of glycoconjugates to near normal concentration in the plasma and buccal mucosa. The protective effect of carnosic acid is probably due to their inhibitory effect on glycoprotein synthesis or on the activity of the enzymes involved in aberrant glycosylation, fucosylation or sialylation of cell surface glycoconjugates. Further studies are therefore warranted to study the effect of carnosic acid on the role of glycosyl, fucosy or sialyl transferases during DMBA induced hamster buccal pouch carcinogenesis.

ACKNOWLEDGEMENT

Financial assistance from Indian Council of Medical Research (ICMR), New Delhi, is gratefully acknowledged.

REFERENCE

Chen, X., Varki, A. Advances in the biology and chemistry of sialic acids. ACS Chem Biol, 5, 2010 pp 163-176.

Cheung, S., Tai, J. Anti-proliferative and antioxidant properties of rosemary Rosemarinus officinalis. Oncol Rep, 17, 2007 pp 1525–1531.

Dabelsteen, E. Cell surface carbohydrates as prognostic markers in human carcinomas. J Pathol, 179, 1996 pp.358-369.

Dische, L. and Shettles, L.B. Specific color reactions of methyl pentoses and spectrophotometric micromethod for their determination. J Biol Chem, 175,1948 pp 595-604.

Folch, J., Lees, M. and Sloane, G.H. A simple method for isolation, and purification of total lipids from animal tissues. J Biol Chem, 226, 1957 pp 497-509.

Glick, M.C. Fucosylation- A role in cell function. In: Walborg EF, ed. Glycoproteins and glycolipids in disease processes. Washington DC: American Clinical Society, 1978 pp 405-411.

Goodarzi, M.T., Shafiel, M., Nomani, H. and Shahriarahmadi, A. Relationship between total and lipid bound serum sialic acid and some tumor markers. Iran J Med Sci 30,2005 pp 124-127.

Johnson, N. Tobacco use and oral cancer: A global perspective. J Dent Educ, 65, 2001, pp 328-339.

Katopodis, N.N. and Stock, C.C. Improved method to determine lipid bound sialic acid in plasma.Res Commun Chem Pathol Pharmacol. 30,1980 pp 171-180.

Kim, Y.S., Kim, Y.W., Siddiqui, B. and Tsao, D. Membrane-associated fucose-containing glycoproteins and glycolipids of cultured epithelial cells from human colonic adenocarcinoma and fetal intestine. Eur J Cancer Clin Oncol, 18,1982 pp 1329-1336.

Macbeth, R.A., Bekesi, J.G. Plasma glycoprotein in various disease states including carcinoma. Cancer Res 22,1962 pp 1170-6.

MacDougall, S.L., Schwarting, G.A., Parkinson, D., Sullivan, A.K. Increased fucosylation of glycolipids in a human leukaemia cell line (K562-clone I) with decreased sensitivity to NK-mediated lysis. Immunology, 62, 1987 pp 75-80.

Manoharan, S., Balakrishnan, S., Vinothkumar, V., Silvan, S. Anti-clastogenic potential of carnosic acid against 7,12-dimethylbenz(a)anthracene (DMBA)-induced clastogenesis. Pharmacol Rep, 62, 2010 pp 1170-7.

Manoharan, S., Kavitha, K., Balakrishnan, S. and Rajalingam, K. Clerodendron inerme protects cellular integrity during 7,12-dimethylbenz[a]-anthracene-induced hamster buccal pouch carcinogenesis. Afr J Trad CAM, 5, 2008 pp 213-222.

Manoharan, S., Padmanabhan, M., Kolanjiappan, K., Ramachandran, C.R. and Suresh, K. Analysis of glycoconjugates in patients with oral squamous cell carcinoma. Clin Chim Acta, 339,2004pp 91–96.

Manoharan, S., Vasanthaselvan, M., Silvan, S., Baskaran, N., Kumar Singh, A., Vinoth Kumar, V. Carnosic acid: a potent chemopreventive agent against oral carcinogenesis. Chem Biol Interact, 188, 2010 pp 616-622.

Miyata, M., Furukawa, M., Takahasi, K., Gonzalez, F.J. and Yamazoe, Y. Mechanism of 7,12- dimethylbenz(a)anthracene induced immunotoxicity: role of metabolic activation at the target organ. Jpn J Pharmacol, 86,2001 pp 302-309.

Narayanan S. Sialic acid as a tumour marker. Ann Clin Lab Sci, 24, 1994 pp 376-384.

Niebes, P. Determination of enzymes and degradation product of glycosaminoglycan metabolism in the serum of health and various subjects. Clin Chim Acta 42, 1972 pp 399-408.

Peng, C.H., Su, J.D., Sung, T,Y., Ho, S.S., Peng, C.C. Supercritical fluid extracts of rosemary leaves exhibit potent anti-inflammation and anti-tumor effects. Biosci Biotechnol Biochem, 71,2007pp 2223-32.

Pugalendhi, P., Manoharan, S., Suresh,K., Baskaran, N. Genistein and daidzein in combination protect cellular integrity during 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in Sprague Dawley rats. Afr J Trad CAM, 6, 2009 PP 94 – 102.

Rajalingam, K.,Renju, G.L. and Manoharan, S. Effect of Clerodendron inerme on erythrocyte membrane integrity during 7,12-dimethylbenz(a)anthracene in-

duced skin carcinogenesis in Swiss albino mice. Asian J Sci Res,1, 2008 pp 246-255.

Rao, V.R., Krishnamoorthy, L., Kumaraswamy, S.V. and Ramaswamy, G. Circulating levels in serum of total sialic acid, lipid-associated sialic acid and fucose in precancerous lesions and cancer of the oral cavity. Cancer Detect Prev 22,1998 pp 237-240.

Schauer, R.Sialic acids as regulators of molecular and cellular interactions. Curr Opin Struct Biol, 19, 2009 pp 507-14.

Senthil, N., Manoharan, S., Balakrishnan, S., Ramachandran, C.R., Muralinaidu, R. and Rajalingam, K. Modifying effects of Piper longum on cell surface abnormalities in 7,12-dimethylbenz[a]anthracene induced hamster buccal pouch carcinogenesis. Int J Pharmacol, 3,2007 pp 290-294.

Thirunavukarasu, C. and Sakthisekaran, D. Influence of sodium selenite on glycoprotein contents in normal and N-nitrosodiethylamine initiated and phenobarbital promoted rat liver tumors. Pharmacol Res, 48, 2003 pp 167-171.

Varki, A., Schauer, R., Sialic acids. In:Cummings R.D,.Esco, J.D., Freeze, H.H., Stanley,P., Bertozzi, C.R.,Hart, G.W.,Etzler, M.E., Editors, Essentials of Glycobiology, 2nd ed. Plainview (NY):Cold Spring harbor laboratory press, 2008 pp 199-217.

Vasanti, R., Rao, L.K., Kumarasamy, S.V. and Ramaswamy, G. Circulating levels in serum of total sialic acid, lipid associated sialic acid and fucose in precancerous lesions and cancer of the oral cavity. Cancer Detect Prev, 22,1998 pp 237-240.

Visanji, J.M., Thompson, D.G., Padfield, P.J. Induction of G2/M Phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels. Cancer Lett, 237,2006 pp 130–136.

Wagner, W.D. A more sensitive assay discriminating galactosamine and glucosamine in mixture. Anal Biochem, 94,1979 pp 369-394.

Wang, J.W., Aambros, R.A., Weber, P.B., Rosano, T.G. Fucosyltransferase and α-L-fucosidase activities and fucose levels in normal and malignant endometrial tissue, Cancer Res, 55, 1995 pp 3654-3658.

Warren, L. Thiobarbituric acid and assay of sialic acid. J Biol Chem, 30,1959 pp 171-180.

Wijeratne, S.S., Cuppett, S.L. Potential of rosemary (Rosemarinus officinalis L.) diterpenes in preventing lipid hydroperoxide-mediated oxidative stress in Caco-2 cells. J Agric Food Chem, 55, 2007 pp 1193–1199.