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LUPEOL PROTECTS ABNORMALITIES IN CELL SURFACE MOITIES DURING 7, 12-DIMETHYLBENZ[A]ANTHRACENE INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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ABSTRACT

Lupeol, a pentacyclic triterpene, possesses diverse pharmacological and biochemical activities including anticancer and antioxidant effects. Abnormalities in the status of glycoconjugates and lipids in the cell results in malignant transformation. The aim of the present study was to investigate the protective effect of lupeol on cell surface glycoconjugates and lipids abnormalities during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinoma was developed in the buccal pouches of golden Syrian hamsters by treating with 0.5% DMBA in liquid paraffin, three times a week, for 14 weeks. The status of glycoconjugates and lipids were measured using specific colorimetric methods. We observed 100% tumor formation with marked abnormalities in the status of glycoconjugates and lipids in hamsters treated with DMBA alone. Oral administration of lupeol at a dose of 50mg/kg bw, completely prevented the formation of tumors and restored the status of glycoconjugates and lipids in hamsters treated with DMBA. The results of the present study thus suggest that lupeol has the potential to protect cell surface abnormalities during DMBA-induced hamster buccal pouch carcinogenesis.

INTRODUCTION: Oral cancer, the fifth most common malignancy worldwide, accounts for 3-4% in Western countries and 40-50% of all cancers in developing countries including India. India has recorded the highest incidence of oral cancer worldwide and approximately 100,000 individuals are suffering from oral cancer in any given year [1]. Long term tobacco smoking, tobacco chewing, betel quid chewing and alcohol consumption play a major role in the pathogenesis of oral cancer [2].

7, 12- dimethylbenz[a]anthracene is commonly preferred to induce oral cancer in the buccal pouch of golden Syrian hamsters. DMBA induced oral tumor in the hamster buccal pouch revealed 70-80% homology with human oral tumor, both morphologically and histologically [3].

The glycoproteins and proteoglycans comprise the extracellular matrix and are the determinant of cellular and biochemical events in the tissues. Cell surface glycoconjugates and sialic acid play an important role in cell-cell communication and cell adhesion. Sialic acids, a nine-carbon keto sugars, play prominent role in the regulation of the immune response as well as in the progression and metastasis of malignant tumors [4]. The status of glycoconjugates is used to diagnose early detection as well as predicting prognosis of the cancerous lesions. Sialic acid or fucose exists as terminal units of oligosaccharides of glycoproteins.

Abnormal glycosylation in the cell surface is recognized as the unique feature of neoplastic transformation. It has been well documented that altered glycosylation, fucosylation and sialylation may play crucial role in the pathogenesis of several cancers including oral cancer [5].

Tumor progression is associated with overexpression of cell surface glycoconjugates, sialic acid and fucose moities. Fucose, a terminal pentose sugar of the glycoprotein chain, has crucial role in slowing the growth rate of tumor cells. Increased fucosylation of glycoproteins is responsible for decreased cell adhesion and uncontrolled growth. Elevated levels of protein-bound hexose, hexosamine, sialic acids and fucose were reported in the plasma and tumor tissues of human and experimental oral carcinogenesis [6, 7].

Lipids have pivotal role in the maintenance of cellular integrity and altered lipid composition in the cell could thus leads to abnormalities in the physico-chemical properties of the biomembranes, contributing to malignant transformation. The molar ratio of cholesterol and phospholipid determines cell fluidity, fragility and membrane permeability. Altered levels of lipids have been documented in several cancerous conditions including oral cancer [8].

Lupeol, an important bioactive constituent of several plants, fruits and vegetables, is a pentacyclic triterpene in nature (**figure 1**). Lupeol has received great attention due to its broad range of biochemical and pharmacological activities. Lupeol exerted anti-genotoxic, anticancer, anti-inflammatory and hepato-protective effects in experimental animal models [9, 10]. Lupeol did not revealed adverse effects and toxicity even at a dose of 2g/kg bw in experimental animals [11]. The protective effect of lupeol on cellular integrity was not reported so far in experimental carcinogenesis.

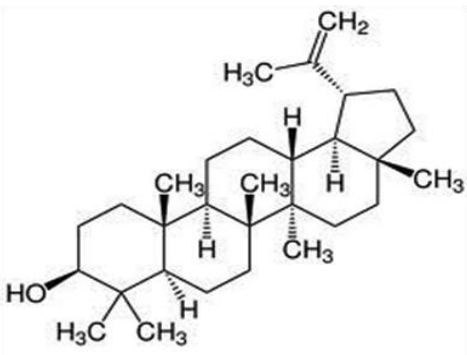

FIGURE 1: CHEMICAL STRUCTURE OF LUPEOL

The present study was therefore designed to study the protective effect of lupeol on cellular integrity by analyzing the status of glycoconjugates and lipids during DMBA-induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Chemicals: DMBA and Lupeol were purchased from Sigma Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade.

Animals: Healthy male golden Syrian hamsters 8-10 weeks old weighing 80-120g were purchased from the National institute of Nutrition, Hyderabad, and were maintained in the central animal house, Rajah Muthaiyah Medical College and Hospital, Annamalai University, Annamalainagar. The animals were housed in polypropylene cages and provided with standard pellet diet and water *ad libitum.* The standard pellet diet is composed of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin, and 55% nitrogen-free extract (carbohydrates). The animals were maintained under controlled conditions of temperature (27±2^{o}C) and humidity (55 ± 5%) with a 12 h light/dark cycle.

Experimental design: The local institutional animal ethics committee (Registration number 160/1999/ CPCSEA) of Annamalai University approved the experimental design. Animals were maintained in accordance with the guidelines of ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use.

Animals were randomly divided in to 4 groups (6 animals per group) on the basis of treatment that each animal received. Group 1 animals served as control and were painted with liquid paraffin (Vehicle) three times a week for 14 weeks on their left buccal pouches. Animals in groups 2 and 3 were painted with a 0.5% solution of DMBA in liquid paraffin three times per weeks for 14 weeks. Group 2 animals received no other treatment. Group 3 animals were orally administered with lupeol at a concentration of 50mg/kg bw three times per week, starting 1 week before the exposure to the carcinogen and continue on days alternate to DMBA application. Group 4 animals were received oral administration of lupeol 50mg/kg bw alone throughout the experimental period.

The experiment was terminated at the end of 15th week and all animals were sacrificed by cervical dislocation.

Samples: Biochemical studies were conducted on plasma and buccal mucosa of control and experimental animals in each group. Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 1,000g for 15 minutes. Tissue samples from animals were washed with ice cold saline and homogenized using Tris-HCl buffer in an all-glass homogenizer with a Teflon® (DuPont, Wilmington, DE) pestle and used for biochemical estimations.

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 glycoproteins. To the dry defatted tissues remaining after lipid extraction, 0.1N H_2SO_4 was added and hydrolyzed at 80ºC for 1 h. 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 in plasma and defatted buccal mucosa was estimated by the method of Niebes *et al* [12].

The protein bound hexosamine in plasma was estimated by the method of Wagner [13]. The total sialic acid estimation in plasma and defatted buccal mucosa were estimated by the method of Dische and Shettles [14]. Lipid extraction from the tissues was done by the method of Folch *et al*., [15]. The total cholesterol and phospholipids in plasma and buccal mucosa were estimated by the method of Parekh and Jung [16] and Zilversmit and Davies [17] respectively.

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: The tumor incidence, tumor volume and tumor burden of control and experimental hamsters in each group are shown in **Table 1**. We have observed 100% tumor formation with mean tumor volume (202.43mm^3) and tumor burden (740.89mm^3) in DMBA alone treated hamsters. Oral administration of lupeol at a dose of 50mg/kg body weight completely prevented the tumor incidence, tumor volume and tumor burden in DMBA treated hamsters. No tumor was observed in control hamsters painted with liquid paraffin alone as well as lupeol alone administered hamsters.

TABLE1: INCIDENCE OF ORAL NEOPLASM IN CONTROL AND EXPERIMENTAL HAMSTERS IN EACH GROUP (n=6)

Parameters	Group I Control	Group II DMBA alone	Group III DMBA + Lupeol	Group IV Lupeol alone
Tumor incidence (oral squamous cell carcinoma)	-	100%	-	-
Total number of tumors/animal	-	22/6	-	-
Tumor volume (mm^3)	-	202.43 ± 18.76	-	-
Tumor burden (mm^3)	-	740.89 ± 68.66	-	-

Tumor volume was measured using the formula, $V = (4/3)\pi[D_1/2][D_2/2][D_3/2]$ where D_1, D_2 and D_3 are the three diameters (mm^3) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors per animal

Figure 2 shows the levels of glycoconjugates in plasma (protein bound hexose, hexosamine, total sialic acid and fucose) and buccal mucosa (protein bound hexose, fucose and total sialic acid) of control and experimental hamsters in each group. The levels of glycoconjugates in plasma and buccal mucosa were significantly increased in hamsters treated with DMBA alone as compared to control hamsters. Oral administration of lupeol to DMBA treated hamsters 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 lupeol alone treated hamsters as compared to control hamsters.

Figure 3 shows the levels of total cholesterol, phospholipids and C/P ratio in plasma and buccal mucosa of control and experimental hamsters in each group. The total cholesterol level and C/P ratio were increased, whereas phospholipids levels were decreased in plasma and buccal mucosa of DMBA alone treated hamsters as compared to control

hamsters. Oral administration of lupeol to DMBA treated hamsters brought back the status of cholesterol, phospholipids and C/P ratio to near normal range. No significant difference was noticed in the levels of plasma and buccal mucosa cholesterol, phospholipids and C/P ratio in lupeol alone treated hamsters as compared to control hamsters.

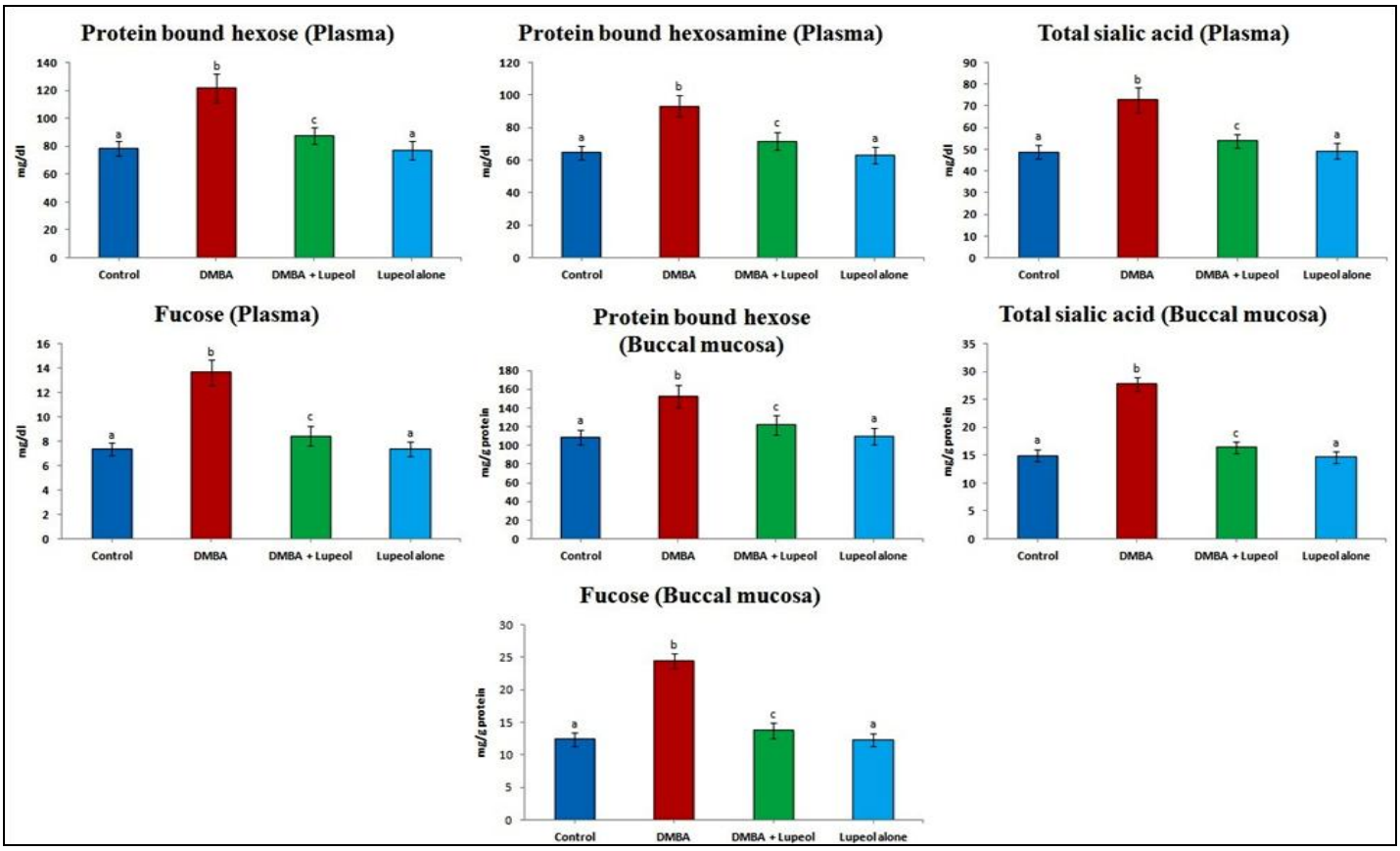

FIGURE 2: PLASMA AND BUCCAL MUCOSA GLYCOCONJUGATES IN CONTROL AND EXPERIMENTAL HAMSTERS IN EACH GROUP

Values are expressed as mean ± SD for 6 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$ (DMRT)

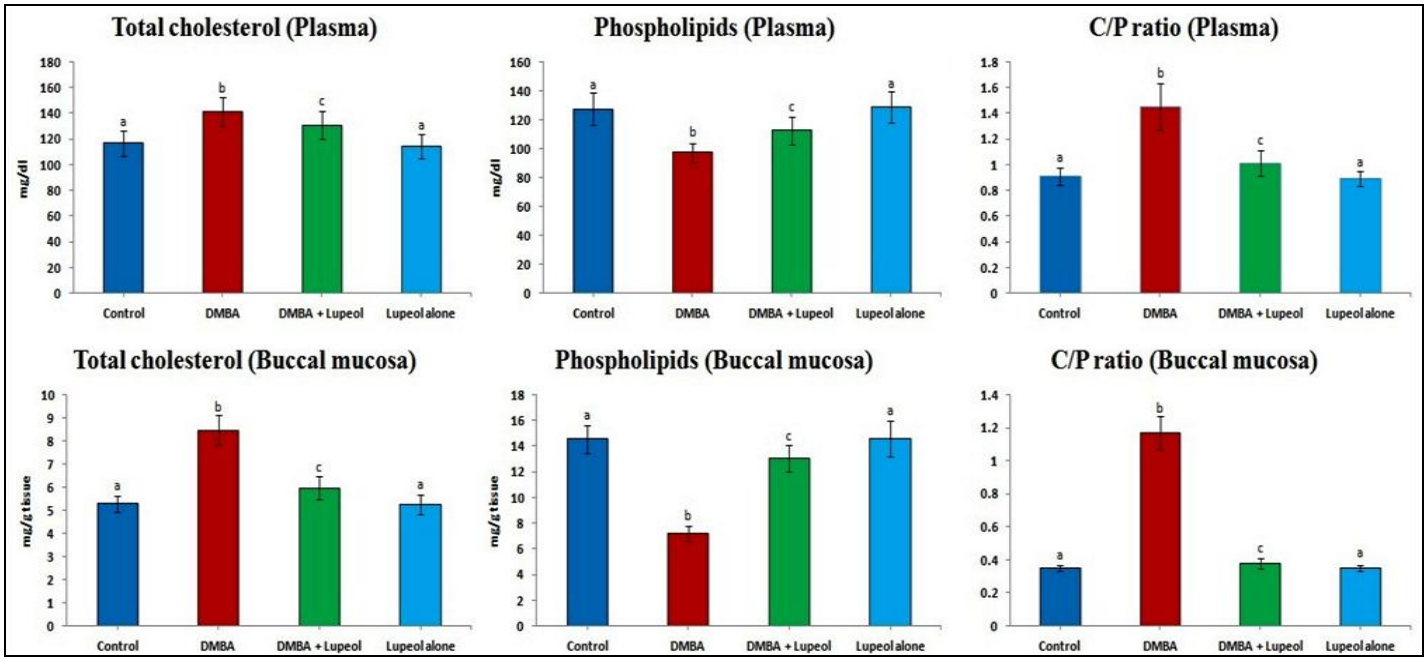

FIGURE 3: PLASMA AND BUCCAL MUCOSA LIPID PROFILE IN CONTROL AND EXPERIMENTAL HAMSTERS IN EACH GROUP

Values are expressed as mean ± SD for 6 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$ (DMRT)

DISCUSSION: The glycosyl residues of glycoconjgates serve an important role in the regulation of cell proliferation and growth. Malignant transformation due to abnormalities in glycoconjugates contributes to aberrant cell-cell recognition, cell adhesion, antigenicity and invasiveness [18]. Glycoconjugates were altered in pre-cancerous and cancerous conditions. The levels of glycoproteins were increased gradually as the tumor progresses from stage I to stage IV of cancer. A positive correlation between glycoproteins content and cell proliferation has been shown in several cancers [19]. Extensive studies reported increased levels of total and lipid bound sialic acids in cancerous conditions [7, 20]. Tumor tissues synthesize specific sialylated sequences during proliferation.

Higher content of total sialic acids, even twice the concentration of normal tissue sialic acids, has been reported in several cancers [21]. Glycoconjugates shedding especially sialic acids and α-galactosyl residues from tumor cell into circulation has been reported in several types of cancers including oral cancer [22]. Over expression of glycoconjugates in the cell surface has been reported in several carcinogenesis including oral cancer [6-8].

Our results are in line with these findings. Enhanced synthesis of glycoproteins in tumor tissues itself and in liver with subsequent release into plasma has been well documented [23]. A large number of experimental studies on carcinogenesis reported that glycoproteins are spontaneously released from tumor cells [24]. Increased levels of glycoconjugates in plasma of DMBA treated hamsters could be due to increased shedding from the tumor tissues.

Tomsik et al [25] reported that L-fucose exhibited promising anticancer potential when administered to tumor bearing mice. Shah et al [26] demonstrated the association of changes in serum fucosylation with oral cancer development. A positive association between plasma fucose level and tumor progression has been reported in experimental carcinogenesis [27]. A large number of experimental and human studies on carcinogenesis suggested that increased turnover and shedding from tumor cells could account for increased plasma sialic acid and fucose content [6, 27].

Our results corroborate these observations. Lipids are utilized as energy fuel not only by normal tissues but also by growing tumor tissues. Abnormalities of the lipid metabolism including hyperlipidemia may be deeply associated with tumor development and progression. A large number of studies reported increased levels of cholesterol in the plasma and tumor tissues [8]. An increase in plasma phospholipids and decrease in tumor tissue phospholipids were also reported [28]. Our results lend credence to these observations.

Tumor tissues may sequester cholesterol from circulation for the biogenesis of new membranes, which in turn accounts for increased total cholesterol in tumor tissues. Increased phospholipid degradation may account for decreased phospholipid content in tumor tissues [29]. Our results support these findings. The cholesterol-phospholipids molar ratio was altered in several cancers including oral cancer [6]. Our results corroborate these observations.

Oral administration of lupeol at a dose of 50mg/kg bw to DMBA treated hamsters not only completely prevented the tumor formation but also brought back the status of glycoconjugates and lipids to near normal range in plasma and buccal mucosa, which suggests that lupeol might have protected the cellular integrity during DMBA-induced hamster buccal pouch carcinogenesis. Although, the exact mechanism for the protective effect of lupeol on cellular integrity is unclear, the possible mechanisms may include its inhibitory effect on the activities of enzymes involved in the glycosylation, sialylation and fucosylation as well as in the lipids synthesis.

Further studies are warranted to elucidate the actual mechanism involved behind the protective effect of lupeol on cellular integrity during DMBA-induced hamster buccal pouch carcinogenesis.

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