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MODULATING EFFECT OF GLYCYRRHIZIN ON THE ACTIVITIES OF CASPASES-3 AND 9, COX-2 AND C-FOS DURING 7, 12-DIMETHYLBENZ (A) ANTHRACENE- INDUCED ORAL CARCINOGENESIS

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Abstract

Oral cancer is the life threatening and disfiguring disease of human population worldwide. Two isoforms of COX are known COX-1 and COX-2. COX-1 levels are in general rather constant in cells whereas COX-2 is synthesized in response to inflammation. COX-2 was found to be expressed at high levels in about one half of colorectal adenomas and most colorectal adenocarcinomas. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Glycyrrhizin, a triterpenoid saponin, is well known for its anti-inflammatory potential in Chinese medicine Licorice root, a traditional herbal remedy, has been used the treatment of several disorders including cancer. Oral administration of glycyrrhizin to hamsters painted with DMBA restored the status of above mentioned molecular markers. The present study thus conclude that glycyrrhizin showed anti-cell proliferative, anti-inflammatory and apoptotic potential during DMBA induced hamster buccal pouch carcinogenesis.

Key Words: Oral cancer, glycyrrhizin, caspase,COX-1 and COX-2.

Introduction

Oral cancer is the cancer of oral cavity which includes lips, tongue, check, floor of mouth, hard and soft palates (Neville *et al.,* 2002). Oral cancer, a part or a group of cancers called head and neck cancers, can develop in any part of the oral cavity or oropharynx. Almost 90% of all oral cancers begin in the squamous cells that cover the surfaces of the over cavity. These cancers are therefore called as squamous cell carcinomas (Scully *et al.,* 2009). While oral cancer accounts for 3-4% of all cancers in western countries this form of cancer accounts for more than 30-40% of all cancers in developing countries. In USA, approximately 35, 000 Americans are affected by oral cancer each year (Lambert *et al.,* 2011). India has recorded highest incidence of oral cancer and this form of cancer accounts for 40-50% of all cancers (Moore *et al.,* 2010).

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Risk factors of Oral cancer

A risk factor is anything that increases the chance of developing a disease. Heavy smokers who use tobacco for a long time are most at risk. The risk is even higher for tobacco users who drink alcohol heavily (Petti, 2009).

Alcohol: People who drink alcohol are more likely to develop oral cancer than people who don't drink. The risk increases with the amount of alcohol that a person consumes (Reidy *et al.,* 2011).

Sun: Cancer of the lip can be caused by exposure to the sun. Using a lotion or lip balm that has a sunscreen can reduce the risk. The risk of cancer of the lip increases if the person also smokes (Ekramuddaula *et al.,* 2011).

Other factors that may increase the risk for oral cancer including, Chronic irritation (such as from rough teeth, dentures, or fillings), Human papilloma virus (HPV) infection, Taking medications that weaken the immune

system (immunosuppressants), Poor dental and oral hygiene, Diet and nutrition (vitamin A deficiency, low fruit and vegetables intake (Warnakulasuriya, 2010).

Symptoms: The most common symptoms of oral cancer includeing, Sore, lump, or ulcer in the mouth, White or red patches in the oral cancer, Chewing problems, Mouth sores, Pain with swallowing, Speech difficulties, Swallowing difficulty, Swollen lymph nodes in the neck, Tongue problems, Weight loss, A sore on the lip, tongue, or other area of the mouth, An ulcer or bleeding (Chowdhury *et al.,* 2010).

Diagnosis: Though various techniques are available to diagnose oral cancer, the most important one used to confirm oral cancer is biopsy. A piece of tissues is removed from the suspected areas and is the analyzed by the oral pathologist under microscope for the presence of cancerous cells (Akbulut *et al.,* 2011). Tests used to confirm oral cancer includeing, Oral biopsy, X-rays and CT scans may be done to determine if the cancer has spread (Groome *et al.,* 2011).

Treatment: Surgery to remove the tumor is usually recommended if the tumor is small enough. Surgery may be used together with radiation therapy and chemotherapy for larger tumors. Surgery is not commonly done if the cancer has spread to lymph nodes in the neck (Bekiroglu *et al.,* 2011).

Prevention: Avoid smoking or other tobacco use, Have dental problems corrected, Limit or avoid alcohol use, Practice good oral hygiene (Da Silva *et al.,* 2011).

Molecular markers of Oral cancer

Cyclooxygenase (COX-2)

Two isoforms of COX are known COX-1 and COX-2.COX-1 levels are in general rather constant in cells whereas COX-2 is synthesized in response to inflammation. COX-2 is an inducible enzyme that catalyzes the rate limiting step in the synthesis of prostaglandins from arachidonic acid. COX-2 was found to be expressed at high levels in about one half of colorectal adenomas and most colorectal adenocarcinomas.Over expression of Cox-2 was reported in oral carcinogenesis (Nishimura *et al.,* 2004).

Caspases

Caspase-3: Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Caspase 3 is a caspase protein that interacts with caspase 8 and caspase 9. The CASP3 protein is a member of the **c**ysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3. The latter can be activated by caspase-3, caspase-8, caspase-9, caspase-10, CPP32 activating protease, granzyme B (Gran B), and others (Ho *et al.,* 2009).

Caspase-9: The aspartic acid specific protease caspase-9 has been linked to the mitochondrial death pathway. It is activated during programmed cell death (apoptosis). Induction of stress signaling pathways JNK/SAPK causes release of cytochrome c from mitochondria and activation of apaf-1 (apoptosome), which in turn cleaves the pro-enzyme of caspase-9 into the active form.Once initiated caspase-9 goes on to cleave procaspase-3andprocaspase-7, which cleave several cellular targets, including poly ADP ribose polymerase. Reduced activities of caspase 3 and 9 were reported in oral cancer (Hakem *et al.,* 1998).

C-Fos: C-Fos is a cellular proto-oncogene belonging to the immediate early gene family of transcription factors.The proto-oncogene C-fos participates in cellular proliferation by encoding a transcription factor, fos that forms homodimers, or heterodimers with the proto-oncogene c-jun (as AP1). The human homolog of the fos oncogene has been mapped to chromosome region 14q21→q31, and is over expressed in a variety of cancers. Transcription of c-Fos is upregulated in response to many extracellular signals, e.g., growth factors(Vairaktaris *et al.,* 2008).

Glycyrrhizin

Glycyrrhizin, a molecule of glycyrrhetic acid and two molecules of glucuronic acid, one of the active principles of licorice. Glycyrrhizin, a triterpenoid saponin, is well known for its anti-inflammatory potential in Chinese medicine (Jancinová *et al.,* 2007). Glycyrrhizin, possesses a sweet taste and sweetness potentiating characteristics' and have been employed industrially for this reason. It also possesses diverse pharmacological effects including anti-ulcer, anti-viral, antioxidant and hepatoprotective potential. Glycyrrhizin also inhibited the tumor formation in diethylnitrosamine induced hepatocarcinogenesis (Rahman *et al.,* 2006). Licorice root, a traditional herbal remedy, has been used the treatment of several disorders including cancer (Rahman *et al.,* 2007).

Materials and Methods

Chemicals

Glycyrrhizin, 7, 12-dimethylbenz [a] anthracene (DMBA), were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. c-fos ELISA kit was

purchased from Uscn Life Science Inc. Wuhan, China. The caspase-3 and -9 colorimetric assay kits were purchased from Biovision, Mountain View, CA, USA. COX activity assay kit was purchased from Cayman Chemical Co., USA. All other chemicals and solvents used were of AR grade.

Experimental Design

A total number of 40 hamsters were randomized into four groups of ten animals in each. 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 bw/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.

Enzyme linked immuno sorbent assay (elisa)

ESTIMATION OF Caspase-3

Caspase-3 activity in the buccal mucosa was measured by using caspase-3 colorimetric assay kit [Biovision, USA] according to the manufacturer's instructions. Caspase-3 activity assay was based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-PNA. The buccal mucosa tissues were homogenized in lyses buffer [50mm HEPES, ph 7.4; 5mm CHAPS and 5mm DTT], and kept in ice for 10 minutes. Homogenate was centrifuged in a micro centrifuge (10,000xg) for 1 minute. Supernatant (cytosolic extract) was transferred to a fresh tube and kept on ice for immediate assay. 50 µl of each sample containing 50-200 µg protein was taken into micro titer plate wells. 50 µl of 2x reaction buffer (containing 10mm DTT) was added to each well. 5 µl of 4mm DEVD-pNA substrate was added to each well and incubated at 37°C for 1-2 hour. After incubation, samples in a microtiter plate were read at 400- or 405-nm in a microtiter plate reader. The concentration of pNA released from the substrate was calculated from the absorbance value. Fold increase was determined by comparing the results with the level of the control. The pNA light emission can be quantified using a spectrophotometer or a micro titer plate reader at 400 or 405nm. Caspase-3 activity is expressed as µmol of pNA formed per minute.

Estimation of caspase-9

Caspase-9 activity in buccal mucosa was measured by using caspase-9 colorimetric assay kit [Biovision, USA] according to the manufacturer's instructions. The buccal mucosa tissues were homogenized in lysis buffer [50mm HEPES, ph 7.4; 5mm CHAPS and 5mm DTT] and kept in ice for 10 minutes. Homogenate was centrifuged in a micro centrifuge (10,000 x g) for 1 minute. Supernatant (cytosolic extract) was transferred to a fresh tube and kept on ice for immediate assay. 50 µl of each sample containing 50 – 200 µg protein was taken into microtiter plate wells. 50 µl of 2x reaction buffer (containing 10mm DTT) was added to each well. 5 µl of 4mm LEHD-pNA substrate was added to each well and incubated at 37^0c for 1-2 hour. After incubation samples in a microtiter plate was read at 400 or 405nm in a micro titer plate reader. The concentration of pNA released from the substrate was calculated from the absorbance value. Fold increase was determined by comparing the results with the level of the control. Caspase-9 activity is expressed as µmol of pNA formed per minute.

Extimation of C-fos

c-fos concentration in buccal mucosa was assayed using c-fos ELISA Kit (Uscn Life Science Inc, Wuhan) according to the manufacturer instructions. The tissue was homogenized in 5 – 10 ml of 1X PBS and stored overnight at ≤ -20°C. Freeze-thaw cycles were performed to break the cell membranes. The homogenates were centrifuged for 5 minutes at 5000xg. The supernatant was used for the assay. Wells for standard, blank and test sample was prepared. 100 µl of reconstituted standard in standard diluents [conc. Range - 0.312, 0.625, 1.25, 2.5, 5, 10 and 20 ng/ml], blank and test samples were added to the appropriate wells. The microtiter plate was covered with plate sealer and incubated for 2 hours at 37°C. After the incubation period, 100µl of Detection Reagent A working solution was added to each well and incubated for 1 hour after covering with plate sealer. After incubation the wells were washed with 400 µl of 1x wash solution. Then 100µl of Detection reagent B working solution was added to each well and incubated for 30 minutes at 37°C. Wash process was repeated and 90 µl of substrate solution was added to each well and incubated for 15 to 20 minutes at 37°C. Then 50µl of stop solution was added to each well. The color developed was read at 450nm in a micro plate reader. The c-fos levels were expressed as ng/ml.

Extimation of COX-2

COX-2 activity was measured by using Cayman's

COX activity assay kit, according to manufacture instructions. The buccal mucosa was rinsed in a Tris buffer, pH 7.4 and homogenized in 5-10 ml of cold buffer (0.1M Tris HCL; pH 7.8 containing 1mM EDTA). It was centrifuged at 10,000xg for 15 minutes at 4°C, and the supernatant was used for assay. 150 µl of each sample was transferred to microcentrifuge tube and then placed in boiling water for 5 minutes. Tubes were centrifuged at 8000xg for 1 minute in a microcentrifuge. The supernatant was used to generate the background values. The wells were marked according to the manufacturer's instruction. Specified volume of assay buffer was added to respective wells. 10 µl of heme was added to all wells. Then 10 µl of COX standard was added in the designated wells on the plate. 40 µl of supernatant obtained from each boiled sample was added to the background wells. 40 µl of respective sample was added to background wells. 10 µl of Dup-697 or Sc 560 was added to specified wells. The plate was incubated for 5 minutes at 25°C. 20 µl of colorimetric substrate was added to each well. 20 µl of arachidonic acid solution was added to the entire well to initiate the reaction. The plate was incubated for 5 minutes at 25°C. The absorbance was read at 590nm using plate reader. The COX-2 activity was expressed as nmol of TMPD oxidized per minute.

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

Buccal mucosa caspase-3 and-9, COX-2 and c-fos status of control and experimental hamsters in each group is shown in Fig. 1-4 respectively. The activities of caspase-3 and -9 were significantly decreased whereas COX-2 andc-fos were increased in hamsters treated with DMBA alone. Oral administration of glycyrrhizin to hamsters treated with DMBA brought back the status of above markers to near normal range. No significant difference was noticed in the status of above markers in control hamsters and hamsters treated with glycyrrhizin alone.

Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$. (Analysis of variance followed by DMRT).

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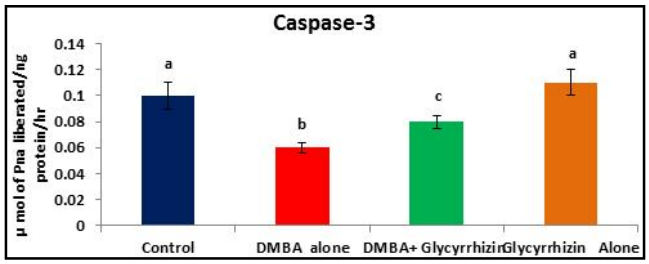

Fig. 1: Activity of Caspase-3 in control and experimental hamsters in each group.

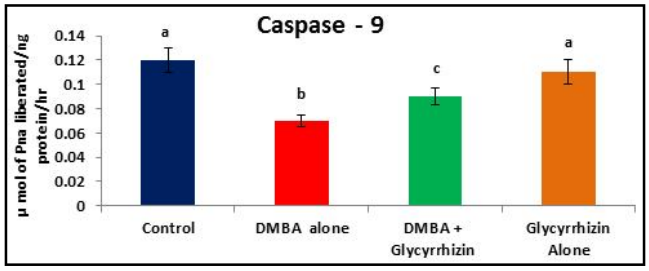

Fig. 2: Activity of Caspase-9 in control and experimental hamsters in each group.

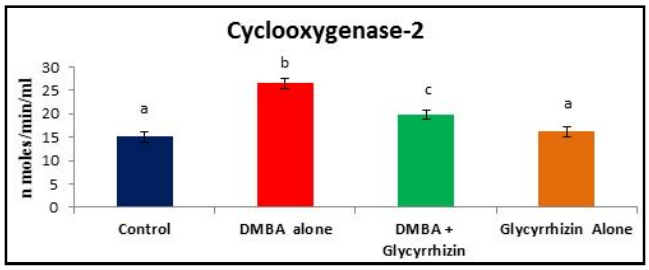

Fig. 3: Activity of COX-2 in the control and experimental hamsters in each group.

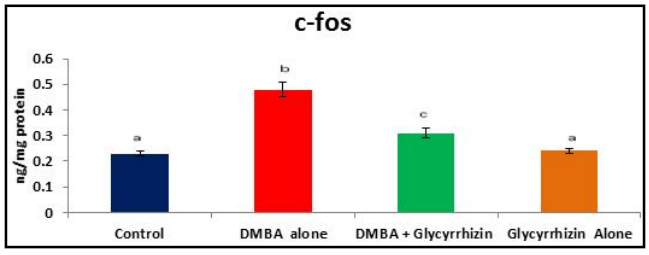

Fig. 4: Activity of c-fos in control and experimental hamsters in each group.

Conclusion

Early diagnosis and prevention of cancer can be achieved by understanding the molecular mechanism associated with the pathogenesis of carcinogenesis. Rapid and abnormal cell-proliferation and defect in apoptotic potential and the central feature of malignancies. The present study has investigated the modulating effect of glycyrrhizin on the activity of caspase-3, 9, c-fos and COX-2 during DMBA induced hamster buccal pouch carcinogenesis. The activities of caspase-3, caspase -9, cox-2 and c-fos were measured in the buccal mucosa of control and experimental animals using ELISA reader. The activities of buccal mucosa caspase-3, caspase -9 were decreased whereas the status of cox-2 and c-fos was increased in tumor bearing animals. Oral administration of glycyrrhizin to hamsters painted with DMBA restored the status of above mentioned molecular markers. The present study thus conclude that glycyrrhizin showed anti-cell proliferative, anti-inflammatory and apoptotic potential during DMBA induced hamster buccal pouch carcinogenesis.

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