RESEARCH COMMUNICATION

Ursolic Acid Attenuates Oxidative Stress-mediated Hepatocellular Carcinoma Induction by Diethylnitrosamine in Male Wistar Rats

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Abstract

Hepatocellular carcinoma is the most common primary cancer of the liver in Asian countries. For more than a decade natural dietary agents including fruits, vegetables and spices have drawn a great deal of attention in the prevention of diseases, preferably cancer. Ursolic acid is a natural triterpenoid widely found in food, medicinal herbs, apple peel and other products it has been extensively studied for its anticancer and antioxidant properties. The purpose of this study was to evaluate the effect of ursolic acid in diethylnitrosamine (DEN) induced and phenobarbital promoted hepatocarcinogenesis in male Wistar rats. Antioxidant status was assessed by alterations in level of lipid peroxides and protein carbonyls. Damage to plasma membrane was assessed by levels of membrane and tissue ATPases. Liver tissue was homogenized and utilized for estimation of lipid peroxides, protein carbonyls and glycoproteins. Anticoagulated blood was utilized for erythrocyte membrane isolation. Oral administration of UA 20 mg/kg body weight for 6 weeks decreased the levels of lipid peroxides and protein carbonyls at a significance of $p<0.05$. Activities of membrane and tissue ATPases returned to normal after UA administration. Levels of glycoproteins were also restored after treatment. Histopathological observations were recorded. The findings from the above study suggest the effectiveness of UA in reducing the oxidative stress mediated changes in liver of rats. Since UA has been found to be a potent antioxidant, it can be suggested as an excellent chemopreventive agent in overcoming diseases like cancer which are mediated by free radicals.

Key Words: Glycoproteins - lipid peroxidation - membrane ATPases - protein carbonyl - *Rosmarinus officianalis*

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Introduction

Hepatocellular carcinoma is a major malignancy world wide (Lodato et al., 2006). It is the fourth most common malignant tumor in the world (Lee et al., 2005) and is the second leading fatal disease in mainland China El-Serrag (2004). Chemical hepatocarcinogenesis is a favourite model in rat, to study the mechanism of biotransformation of a normal cell to malignant populations.

Carcinogenesis is a multistep and multistage process that begins with irreversible, but heritable damage to a single cell Dragon (1994). Model systems in animals exhibit this property of cancer development in several organ systems Pitot (1986). Rat liver is one of the most extensively studied models of carcinogenesis and multiple formats have been described for the analysis of cancer development (Goldsworthy et al., 1985). Cell proliferation appears to play a crucial and critical role in several steps in cancer development.

Diethylnitrosamine (DEN) is a representative chemical of a family of carcinogenic N- nitroso compounds and is normally used as a carcinogen to induce cancer in animal models (Bhosale et al., 2002). Diethylnitrosamine is an environmental carcinogen and hepatotoxin. It causes degenerative, proliferative and neoplastic lesions in liver (Qi et al., 2008). DEN is a chemical agent that can alkylate DNA molecule with itself being converted to highly reactive molecule by P-450 dependent oxygenases (Li, 2005).

NDEA has been found in a variety of products that would result in human exposure, including main stream tobacco smoke. DEN is bioactivated by P-450 mediated _-hydroxylation producing _-hydroxyl nitrosamine. It is hydroxylated principally by ethanol inducible CYP2E1 in liver. DNA-adduct formation proceeds through an ethyldiazonium ion intermediate and evolution of N2 (Michejda et al., 1982; Singer and Grunberg, 1983). DEN administration influences cytotoxicity, cell proliferation and DNA replication rates (Verna et al., 1996). When administered in drinking water, *N*-nitrosodiethylamine induced liver tumors in guinea pigs, rabbits, dogs, and rats.

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The administration of such carcinogenic substances brings about changes in enzyme levels arising from clonic proliferation of cells (Kang et al., 1997). Phenobarbital is an anti-epileptic drug. It is a commonly employed non-genotoxic agent to induce liver tumor in lab animals Cunningham (1996). It promotes hepatocarcinogenesis when administered after an initiating dose of DEN (Loppen et al ., 2002).

Studies on natural products are on rise not only because of their medicinal value, but also because of their lesser side effects. Triterpenoids are a class of naturally occurring compounds that are found in a variety of European plants & fruits. They are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antitumor, immunomodulatory and tonic effects. Ursolic acid (3ß hydoxy urs-12 en – 28 oic acid) is a triterpenoid found widespread, especially in higher plants like Rosmarinus officianalis.

It is shown to lower COX-2 transcription (Subbaramaiah et al., 2000). It has been studied for its beneficial effects on liver in CCl4 toxicity model (Martin-Aragon et al., 2001) and antihepatoma activity in mice. It has also been reported that UA has potent antiproliferative activity against HepG2 human liver cancer cell line (Zen et al., 2006). The current study has been focused to understand the antioxidant effect of UA in DEN induced hepatocarcinogenesis model in male Wistar rats. Lipid peroxidation and protein carbonyl in liver tissues were studied to assess the free radical mediated oxidative stress and glycoprotein and membrane ATPases were estimated to reflect the membrane damage of the organ.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN), phenobarbital and ursolic acid (UA) were procured from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used for the experiments were of analytical grade.

Animals

Male Wistar albino rats weighing approximately 100 -150 g were divided into five groups of 6 rats each. All rats were maintained in Polypropylene cages with 12 h light/dark cycle under constant temperature and humidity. Animals were fed a commercial diet and had ad libitum access to food and water. The research was approved by the institutional animal ethical committee (IAEC NO.02/078/07).

Experimental design

Group 1: Control animals given vehicle alone (0.2% gum acacia for 34 weeks).

Group 2: Animals induced with DEN by a single intraperitoneal injection
(200mg/kg b.wt in saline). After 2 weeks recovery period, the carcinogenic effect was promoted by Phenobarbital at 0.6% finely ground with 0.2% gum acacia and was given in drinking water for 26 weeks.

Group 3: Animals were treated with Ursolic Acid at the dosage of 20mg/kg b.wt in 0.2% gum acacia per day for 15 days before administration of DEN as in Group 2.

Group 4: Animals were treated with Ursolic acid at a dosage similar to Group 3 for 6 weeks after 26 weeks (induction period).

Group 5: Animals were treated with Ursolic acid alone for 6 weeks at the above mentioned dosage to observe the toxicity present (if any) for Ursolic acid.

Protocols

After the experimental period, the animals were killed by cervical decapitation. Blood and liver tissues were collected and washed in ice cold saline (0.89%). The tissues were then blotted to dryness and 10% homogenate was prepared immediately using Tris-HCl buffer 0.1M (pH-7.4) using a Potter Elvejhem glass homogenizer and the homogenate was used for estimation of lipid peroxides (Ohkawa et al., 1979) & protein carbonyls (Levine et al.,1990). The blood sample collected with 5% EDTA was used to isolate erythrocyte membrane according to the method of Dodge et al., (1963) with slight modifications of Quist, 1980.

Glycoproteins from the liver tissue were extracted with chloroform and methanol. The extract was hydrolysed with acid and finally used for the estimation of hexose, after Neibes (1972), hexosamine, after Wagner (1979), and sialic acid, after Warren (1959). Na^+K^+ ATPase (E C 3.6.1.3) were estimated by the method of Bonting (1970). The activity of Ca^{++} ATPase (E C 3.6.1.3) was assayed by the method of Hjerten and Pan (1983). The activity of Mg^{++} ATPase (E C 3.6.1.3) was assayed by the method of Ohnishi et al (1982). Inorganic phosphorus was estimated by the method of Fiske and Subba Row (1925). Protein content was estimated by Lowry et al (1951).

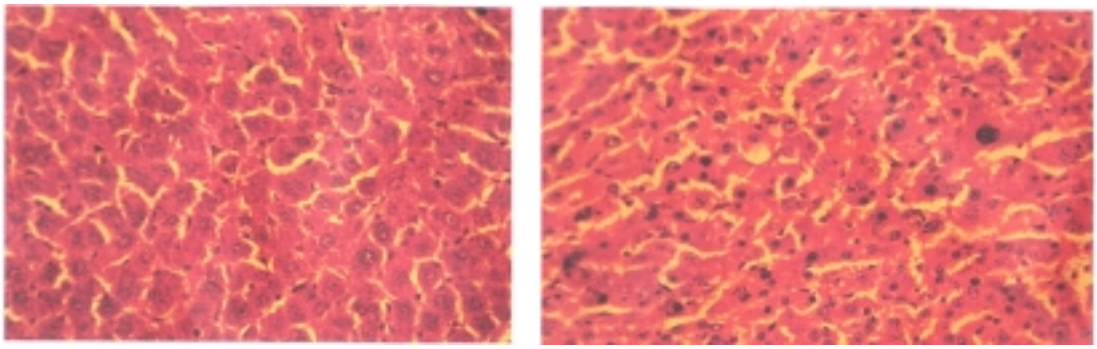

Figure 1. Histopathology of Liver Samples. a) Normal; b) DEN + PB. Note variation in cell size and proliferation

Table 1. Levels of ATPases

Enzymes	Group I	Group II	Group III	Group IV	Group V
Liver					
Na^+/K^+	5.93 ± 0.75	3.65 ± 0.38^{a*}	4.54 ± 0.44$^{b\#}$	4.32 ± 0.50$^{c\$}$	5.37 ± 0.52^{ns}
Ca^{++}	6.63 ± 0.72	4.31 ± 0.42^{a*}	5.89 ± 0.79$^{b\#}$	5.72 ± 0.61$^{c\$}$	6.36 ± 0.82^{ns}
Mg^{++}	10.71 ± 1.4	5.41 ± 0.57^{a*}	9.74 ± 1.02^{b*}	7.15 ± 0.68$^{c\#}$	10.07 ± 0.93^{ns}
Erythrocyte membranes					
Na^+/K^+	1.78 ± 0.19	0.97 ± 0.08^{a*}	1.39 ± 0.17^{b*}	1.17 ± 0.14$^{c\$}$	1.73 ± 0.19^{ns}
Ca^{++}	4.11 ± 0.50	2.85 ± 0.25^{a*}	3.94 ± 0.38^{b*}	3.64 ± 0.32$^{c\$}$	4.14 ± 0.44^{ns}
Mg^{++}	1.91 ± 0.19	0.77 ± 0.09^{a*}	1.76 ± 0.23^{b*}	1.02 ± 0.08$^{c\#}$	1.83 ± 0.17^{ns}

Values are mean ± SD for n=6 rats in each group. Units are expressed as pi liberated /min/mg protein; [a]as compared with group I; [b]groupIII with group II; [c]groupIV with group II; * $p<0.001$, [#]$p<0.01$, [$]$p<0.05$; [ns]Non-Significant

Statistical Analysis

The data were expressed as mean ± standard deviation. Significant changes between the groups were detected by one way analysis of variance (ANOVA) and least significant difference (LSD) method was used to compare the means of different groups. Commercial software SPSS version 10.0 was used for statistical analysis.

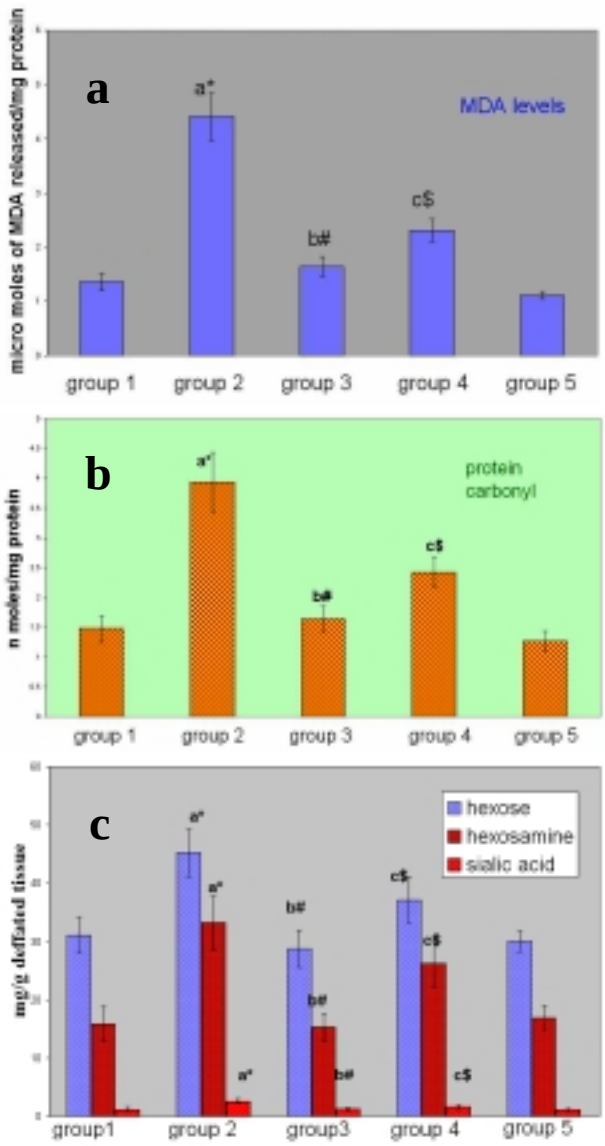

Figure 2. Liver Biochemical Data. a) Lipid Peroxidation; b) Protein Carbonyls; Glycoproteins. Values are mean ± SD for n=6 rats in each group. Group 1, controls; Group 2, DEN+PB; Group 3, UA pretreated; Group 4,UA treated after 26 weeks; Group 5- UA alone; [a]comparison with group 1; [b&c] comparison with group 2;*, #, $, statistically significan at $p<0.001$, $p<0.01$, $p<0.05$, respectively

Results

Histopathology

The liver samples of DEN treated animals showed hyperchromatism, hyperplasia, and proliferating hepatocytes (see Figure 1). On treatment with Ursolic acid the architecture of the liver was restored showing regeneration of normal hepatocytes.

Antioxidant status

There was a significant increase in the levels of lipid peroxidation in cancer bearing animals (group 2) when compared to control animals (group1) with a significance of $p<0.001$ (see Figure 2a). Levels were decreased in UA treated animals (group 4) to approximately 52%. In pretreated animals (group 3), the levels decreased as compared to cancer bearing animals & was closer to control animals, due to the continuous administration of UA. No significant change was seen in UA alone treated animals (group 5).

Regarding levels of protein carbonyls formed during carcinogen and UA treatment, there was a significant increase in cancer bearing animals (group 2), which was reduced by 61% in UA treated animals (group 4)(see Figure 2b). Pretreated animals (group 3) also showed a significant decrease, when compared to cancer bearing animals. There were no marked changes in UA treated animals (group 5).

Membrane damage

Glycoproteins (hexose, hexosamine & sialic acid) were increased significantly in cancer bearing animals (group 2) with a significance of $p<0.001$, which decreased on treatment with UA (see Figure 2c). The results were expressed at significance of $p<0.05$.

Table 1 shows change in the levels of ATPases in liver and erythrocyte membranes of male Wistar rats. The enzyme activities of Na+K+ and Mg++ ATPases were significantly decreased in liver & erythrocyte membrane ($p<0.001$) in carcinogen treated animals (group 2). The levels were restored in UA treated (group 3) and (group 4) animals ($p<0.01$ & $p<0.05$) respectively. Decrease in Ca++ ATPase activities was observed in the erythrocyte membrane of cancer bearing animals (group 2) and increased significantly in group 3 and group 4 animals.

Discussion

Lipid peroxidation is the most extensively studied free-radical mediated process. Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity on a free radical. These free radicals derived from oxygen represent the most important class of such species generated in living systems (Valko et al., 2004). Oxidative stress is discussed as a possible cause of hepatocarcinogenesis in rodents and is suggested to result from excessive production of H2O2 from degradation of fatty acid (Wolfgang et al., 1997).

Lipid peroxidation has been shown to perturb the bilayer structure and modify membrane fluidity (Mandal, 1980; Chattrejee, 1988; Kunimoto, 1981). In recent years much research has been dedicated in identifying the plant components which contribute in combating the oxidative stress and free radical induced damage, which mainly is the first step to chemical carcinogenesis. In the current study, the focus was to assess the levels of oxidative stress markers, lipid peroxides and protein carbonyls in the liver of DEN induced male Wistar rats.

DEN is the principal nitrosamine among environmental carcinogens in intercalating with membrane lipids for free radical formation (Nakae et al., 1997). These radicals cause damage to cellular processes, there by causing decrease of enzyme activities etc leading to cell death. This was evident from our current study too where group 2 cancer bearing animals showed a statistically significant increase in the levels of lipid peroxidation products. The reduced levels of lipid peroxides in group 3 and group 5 rats treated with Ursolic acid indicated the antioxidant property of this compound in reducing the lipid peroxides level henceforth preventing initiation and propagation process by scavenging free radicals and therefore its role in combating oxidative stress. Proteins are important targets of oxidative modifications. Protein carbonyl is a product of irreversible non enzymatic oxidation or carbonylation of protein and indicators of free radical generation in cells (Dalle-donne et al., 2006). Oxygen radicals generated as byproducts of cellular metabolism or from carcinogenic assault result in functional changes in structural & enzymatic proteins Stadtman (1992). The presence of carbonyl group has been used as a marker of reactive oxygen mediated protein oxidation (Berlett and Stadtman, 1997). There was a significant increase in the levels of protein carbonyls in DEN induced animals which was restored to lower levels on treatment with UA. The fact that UA has shown to decrease oxidative stress or MDA products by increasing the antioxidant enzyme status such as catalase (Kim et al., 1996) and thiol status (Martin-Aragon et al., 2001) of the cell might also be a reason for decreased protein carbonyl levels, since carbonyl groups in proteins are mainly introduced by malondialdehydes produced during lipid peroxidation.

Glycoproteins are proteins that contain oligosaccharide chains (glycans) covalently attached to their polypeptide chain. Compositional analysis following acid hydrolysis is one method of identifying sugars, qualitatively and quantitatively. Sialic acid, one of the glycoprotein components is used as a tumor marker Crook (1993). They have a central role of functioning in biological systems such as stabilizing the conformation of glycoproteins on cellular membranes, assisting in cell-cell recognition, and interaction, and serving as chemical messengers in body fluids & tissues Kurtul (2004). Elevated levels have been reported in alcoholics Kurtul (2004) and (Ponnio et al., 1999) and smokers (Lindberg et al., 1991). In animal study, carcinogen administration tends to increase Sialic acid levels (Dich et al., 1997).

Serum glycoproteins have shown to be elevated in lung, prostrate, bladder and GI system (Erbil et al., 1985) and (Kokoglu, 1992). In our current study, there was a significant increase in the levels of glycoproteins in cancer bearing animals (group 2) which suggests the harmful effects of DEN promoted by phenobarbital.

We have observed a significant decrease in the levels of hexose, hexosamine & sialic acid in animals treated with UA suggesting the antitumor nature of the drug because the first reactions of organism to carcinogenic compounds take place at the cellular level, before the effect become visible at higher levels of biological organizations. Since the levels of glycoprotein were reduced in group 3 & group 5 animals, it may be attributed to the protective efficacy of the triterpenoid, UA.

Liver plasma membrane plays a major role in hepatobiliary transport of biliary components and xenobiotics. There is a need for healthy signaling and message flow to maintain a good communication and coordination of biochemical process within the cell. Alterations in the plasma membrane of liver in its composition and fluidity can influence cancer mediated transport process and membrane bound enzyme activities (Ana et al., 1999). Injury to cell membrane by free radicals has been a recent focus since the vital activities of the cell are challenged. The three important ATPases of the plasma membrane are the Na^+K^+ATPase, Ca^{++} ATPase and Mg^{++} ATPase. Na^+K^+ATPase uses energy derived from the hydrolysis of ATP to keep a high K^+ and a low Na^+ concentration in the cytoplasm which in turn provides the driving force for the net movement of other substance such as Ca^{++}, aminoacids and H+ (Contreras et al.,1999).

Decrease in the activity of Na^+K^+ ATPase and Mg^{++} ATPase occurs during tumor growth particularly in malignancy. This is well correlated with the current study wherein a similar decrease in the activities were found in cancer bearing animals (group 2), which suggests the condition of malignancy and progression of cancer. The decreased activity might also be due to lipid peroxides induced by DEN which could have altered membrane structure. In the current study significant increase in the activities of these two enzymes in group 3 & group 5 animals suggest that decrease in levels of lipid peroxides could have contributed to and increase in the enzyme activities, indicating their protective role in maintaining membrane integrity.

Ca^{++} ATPase activity was found to be decreased in cancer bearing animals. Free intracellular calcium, acting as a second messenger, is crucial for a diverse range of

biological functions (Berridge et al., 2000). Intracellular calcium signaling is also a key regulator of proliferation Lipskaia (2004), cell cycle progression and apoptosis Orrenius (2003). The plasma membrane Ca^{++}ATPase (PMCA) or pump belongs to the family of P-Type ATPases and is a critical regulator of free intracellular Ca^{++}. There are two isoforms of PMCA (PMCA1-4) Carafoli (1994). PMCA alterations are also found to be associated with tumorigenesis. The decrease in the activity of cancer bearing animals suggest that there is a high concentration of Ca^{++} inside the cells due to toxicity created by DEN, which the calcium pump tries to eliminate, to keep its level low. Subsequent increase in the activity was recorded after treatment with UA suggesting its protective role. Further Ca^{++} ATPase activity is mainly impaired due to oxidative modification of thiol groups present in this enzyme which in turn is due to the generation of free radicals (Jain and Sohet, 1981). The fact that UA has been found to reduce protein carbonyls in our study thereby reducing stress and free radical formation might be a reason in restoring the enzyme activity. It can be deciphered that ursolic acid conditions hepatic cells, preventing any further damage to liver parenchyma which in turn would have decreased the leakage of enzymes into circulation.

Further, since ursolic acid has been reported to have high binding capacity to liver, it can be assumed that quicker regeneration of hepatocytes and hepatic parenchyma would have occurred on administration of ursolic acid thus increasing the functional efficiency of the liver.

From the above study, it can be concluded that Ursolic acid has the capacity to modulate the activities of membrane bound enzymes and decrease the levels of lipid peroxides, glycoproteins and protein carbonyls thus restoring the membrane integrity and suggesting its role as a chemopreventive anticancer and antioxidant compound.

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