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Research Article

UMBELLIFERONE WITH VITAMIN C MODULATES LIPID PROFILE INDICES IN DIETHYLNITROSAMINE INDUCED HEPATOCELLULAR CARCINOMA

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ABSTRACT

Umbelliferone (UMB) and vitamin C are naturally available antioxidants with the capacity to inhibit the formation of tumors in several cancer models. In the present study, the authors have investigated if the combination of UMB and vitamin C had any role in lipid metabolism in diethylnitrosamine (DEN) induced hepatocellular carcinoma (HCC) in rats. Rats were randomly distributed into 6 groups of 6 rats each. Rats in group 1 received standard pellet diet and served as controls. HCC was induced in Group 2 rats by providing 0.01 % DEN through drinking water for 15 weeks. Group 3 rats received UMB via intra gastric intubation at a daily dose of (30 mg/kg body weight) + vitamin C (200 mg/kg body weight) through drinking water for 16 weeks. Group 4 rats were pretreated with UMB + vitamin C, 1 week prior to the administration of DEN and it was also continued until the end of the experiment. Group 5 rats were post-treated with UMB + vitamin C for 5 weeks after the administration of DEN for 10 weeks and further continued until the end of experiment. Group 6 rats were treated with UMB + vitamin C along with DEN for the entire 15 weeks of experimental period. DEN-induced rats showed increased levels of total cholesterol and triglycerides and decreased levels of phospholipids and free fatty acids. These changes were reversed to near normal levels in groups treated with UMB + vitamin C. The histopathological and ultra-structural studies were performed to further confirm the occurrence of apoptotic morphological changes at the cellular level.

Keywords: Diethylnitrosamine, hepatocellular carcinoma, umbelliferone, vitamin C, lipid profile.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most life-threatening human cancers with more than one million fatalities occurring annually and worldwide[1]. Hepatitis viral infections, fungal toxins, toxic industrial chemicals, air and water pollutants are the major risk factors implicated in liver cancer. Lack of effective diagnostic tools for early detection and limited treatment options contribute to the high mortality associated with this disease. An urgent need exists for the discovery and development of novel, preventive as well as therapeutic strategies to combat the current morbidity and mortality associated with HCC. Therefore, an alternative or complementary effective approach for secondary prevention needs to be identified in combination with nutritional interventions. UMB is a major bio transformed product of coumarin (1, 2-benzopyrone). As an antioxidant, it possesses promising antidiabetic[2], antihyperglycemic[3] spasmolytic and antitumour[4] properties. It is used in the synthesis of various drugs, especially anticancer medications. The authors have reported earlier that UMB has anticancer properties in DEN induced HCC and DMH induced colon cancer[5,6]. DEN is a hepatocarcinogen, normally used as a carcinogen to induce liver cancer in animal models[7]. Lipids form a diverse group of water-insoluble molecules that include cholesterol, phospholipids, triacylglycerides, and free fatty acids. Cholesterol is biosynthesized by all animal cells because it is an essential structural component of animal cell membranes that is required to maintain both membrane structural integrity and fluidity[8]. Phospholipids are a major component of all cell membranes as they can form lipid bilayers[9]. Fatty acids are usually derived from triglycerides or phospholipids and they are consumed by the mitochondria to produce ATP through beta oxidation[10]. Triglyceride is an ester derived from glycerol and fatty acids and they are important in the control of LDL and HDL cholesterol levels. The present study was conducted to delineate the role of hyperlipidemia during DEN-induced hepatocarcinogenesis in Wistar male albino rats.

MATERIALS AND METHODS

Chemicals

UMB and DEN were purchased from Sigma-Aldrich Chemical Company, Saint-Louis, MO, USA. All the other chemicals and reagents used were of analytical grade.

Tumor induction

HCC was induced in male Wistar rats by administering DEN at 200 mg/kg body weight in drinking water for 16 weeks[11].

Formulation and administration of UMB and vitamin C

UMB was freshly prepared and dissolved in 10 % dimethyl sulphoxide (DMSO)[12] at a daily dose of 30 mg/kg body weight. Vitamin C was given orally through

drinking water daily at a dose of 200 mg/kg body weight for 16 weeks[13].

Animal housing and diets

Male Wistar albino rats weighing about 150-180 g were obtained from Sri Venkateshwara Enterprises, Bangalore, India. After 1 week of acclimatization, all rats were housed in groups of six per polypropylene plastic cage, covered with metal grids and containing a hygienic bed of husk in a specific-pathogen free animal room under controlled conditions with a 12-hours light/12-hours dark cycle. They were provided with standard food pellets supplied by Hindustan Lever Ltd, Mumbai, India, and tap water *ad libitum*. The study was conducted after obtaining clearance from the Institutional Animal Ethics Committee (Reg. No. P. Col/63/2011/IAEC/VMCP) of Vinayaka Mission College of Pharmacy, Salem, Tamil Nadu, India.

Study design and treatment schedule

Rats were randomly distributed into six groups of six rats each. Rats in group 1 received standard pellet diet and served as controls. HCC was induced in Group 2 rats by providing 0.01 % DEN through drinking water for 15 weeks. Group 3 rats received UMB via intra gastric intubation at a daily dose of (30 mg/kg body weight) with vitamin C through drinking water (200 mg/kg body weight) for 16 weeks. Group 4 rats were pretreated with UMB (30 mg/kg body weight) and vitamin C (200 mg/kg body weight), 1 week prior to the administration of 0.01 % DEN and this was continued till the end of the experiment. Group 5 rats were post-treated with UMB (30 mg/kg body weight) and vitamin C (200 mg/kg body weight) for 5 weeks after the administration of DEN for 10 weeks and further continued till the end of experiment. Group 6 rats were treated with UMB (30 mg/kg body weight) and vitamin C (200 mg/kg body weight) along with DEN for the entire 15 weeks of experimental period. At the end of the experimental period, the rats were sacrificed by cervical dislocation and the blood samples and liver tissue of the animals were taken for analysis.

Preparation of tissue homogenate and histopathological studies

Liver tissue was removed immediately and washed with ice-cold saline and homogenized in the appropriate buffer in a tissue homogenizer. Histological sections stained with hematoxylin and eosin was used to confirm the presence and type of tumors by histopathological examination.

Biochemical Estimation

Lipids from the tissues were extracted by the method of Folch *et al*.[14] Total cholesterol was determined by the method of Zlatkis *et al*.[15] Phospholipids were determined by the method of Zilversmit and Davis[16]. Triglycerides were determined by the method of Fossati and Lorenzo[17]. Free fatty acids were determined by the method of Falholt *et al*[18].

Ultra structure studies by transmission electron microscopy

The liver samples were fixed in Karnovsky's fixative immediately after euthanization of rats for 6-8 hours at 4ºC. These samples were post-fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 2 hours at 4ºC, dehydrated in ascending grades of acetone, infiltrated and embedded in araldite CY212 and polymerized at 60ºC for 72 hours. Thin (60-70 nm) sections were cut with an ultra-microtome. The sections were mounted on copper grids and stained with uranyl acetate and lead citrate and observed under a transmission electron microscope.

RESULTS

Microscopic observations of liver in control and experimental rats

Histological examination of liver sections from control and drug control rats (groups 1 and 3) revealed normal architecture (Figure 1A and 1C). DEN induced rats (group 2) showed tumor cells which were smaller than normal cells with granular cytoplasm and large hyper chromatic nuclei (Figure 1B). But pre, post and entire period treated rats (groups 4, 5 and 6) showed fewer neoplastically transformed cells and the hepatocytes maintained near normal architecture (Figure 1D, 1E and 1F).

Effect of UMB with vitamin C on lipid profile of serum and liver tissue of control and experimental rats

Figure 2 shows the levels of lipid components - cholesterol, phospholipids, triglycerides, and free fatty acids in the serum of control and experimental rats. DEN induced (group 2) rats showed a significant increase in the levels of cholesterol and triglycerides with subsequent decrease in the levels of phospholipids and free fatty acids when compared with control rats (group 1). On the other hand, the pre, post and entire period treated rats (groups 4, 5 and 6) showed a significant decrease in the levels of cholesterol and triglycerides with subsequent increase in the levels of phospholipids and free fatty acids when compared with DEN induced rats. Figure 3 shows the levels of cholesterol, phospholipids, triglycerides and free fatty acids in the liver tissue of control and experimental rats. DEN induced rats (group 2) showed a significant increase in the levels of cholesterol and triglycerides with subsequent decrease in the levels of phospholipids and free fatty acids in liver when compared with controls (group 1). But pre, post and entire period treated rats (groups 4, 5 and 6) showed a significant decrease in the levels of cholesterol and triglycerides with subsequent increase in the levels of phospholipids and free fatty acids when compared with DEN induced rats.

Effect of UMB with vitamin C on the ultra structural changes in the liver tissue of control and experimental rats

Figure 4 shows ultra structural changes in control and experimental rats. DEN induced rats (group 2) showed cells with multiple dysplastic nuclei close to each other with irregular shaped cytoplasm and defragmented nucleolar contents. Control and drug control rats (groups 1 and 3) showed normal liver architecture, nuclei and cytoplasm. The pre, post and entire period treated rats (groups 4, 5 and 6) showed changes of apoptosis and liver cells with shrunken nucleus and condensed chromatin undergoing apoptosis.

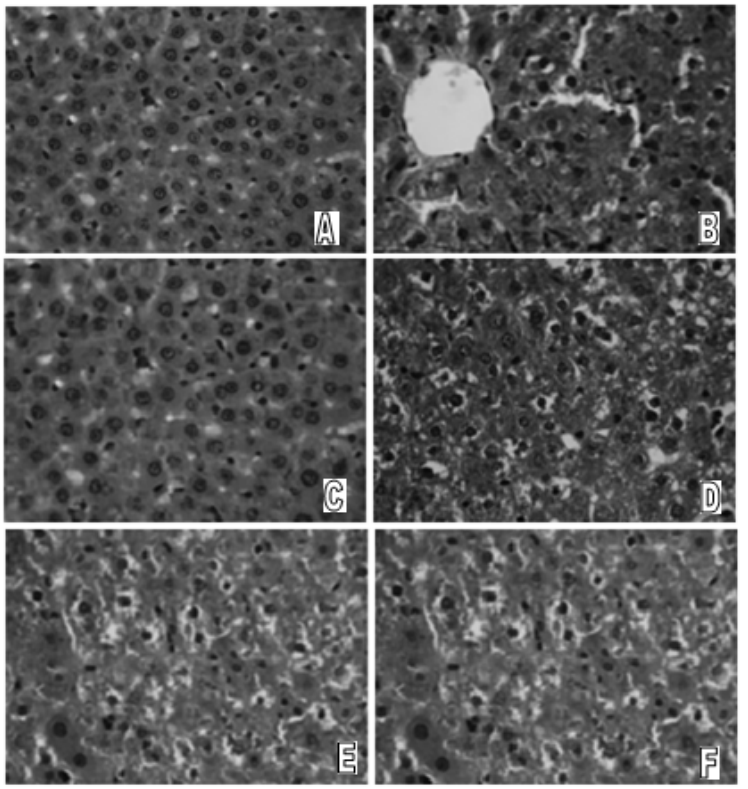

Figure 1: Microscopic observations of liver in control and experimental rats

Histological examination of liver sections from control and drug control rats (group 1 and group 3) revealed normal architecture and cells with granulated cytoplasm and small uniform nuclei (Figure 1A and 1C). DEN alone induced rats (group 2) showed loss of architecture and tumor cells which were smaller than normal cells with granular cytoplasm and large hyperchromatic nuclei (Figure 1B). Further, pre-treated, post-treated and entire period treated rats (group 4, 5 and 6) showed fewer neoplastically transformed cells and the hepatocytes maintained near normal architecture (Figure 1D, 1E and 1F)

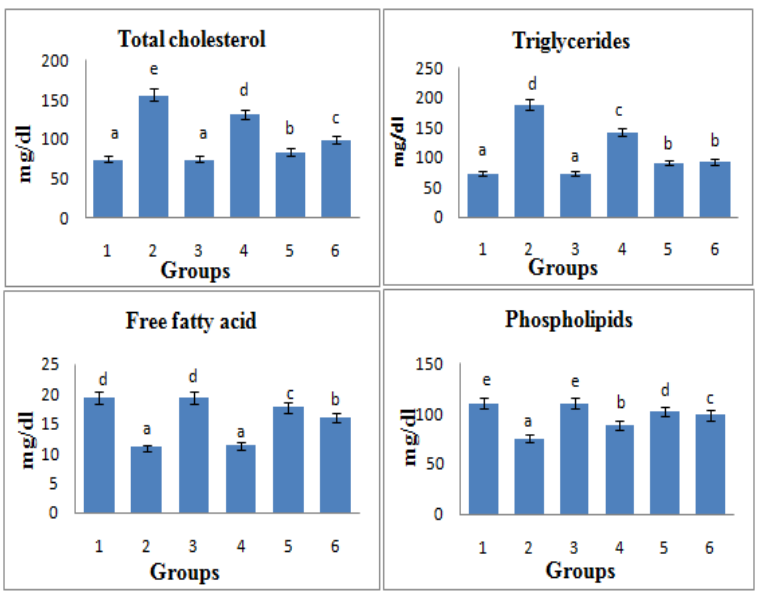

Figure 2: Effect of UMB and vitamin C on hepatic serum lipid profiles of control and experimental rats

Data presented as the means ± SD of each group. [a–e] $p < 0.05$ the values not sharing a common superscript letter are significantly different from the DEN induced groups (analysis of variance followed by DMRT)

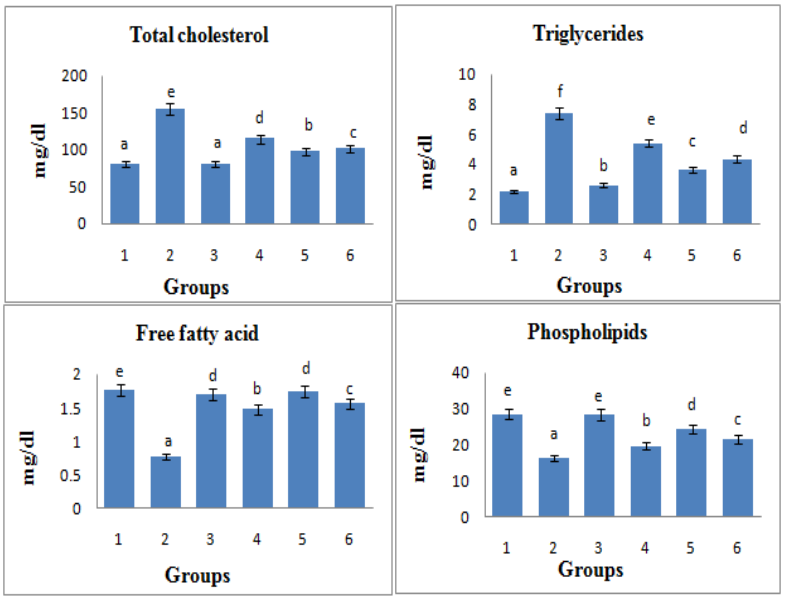

Figure 3: Effect of UMB and vitamin C on hepatic tissue lipid profiles of control and experimental rats

Data are presented as the means ± SD of each group. $^{a-f}P < 0.05$ the values not sharing a common superscript letter are significantly different from the DEN induced groups (analysis of variance followed by DMRT)

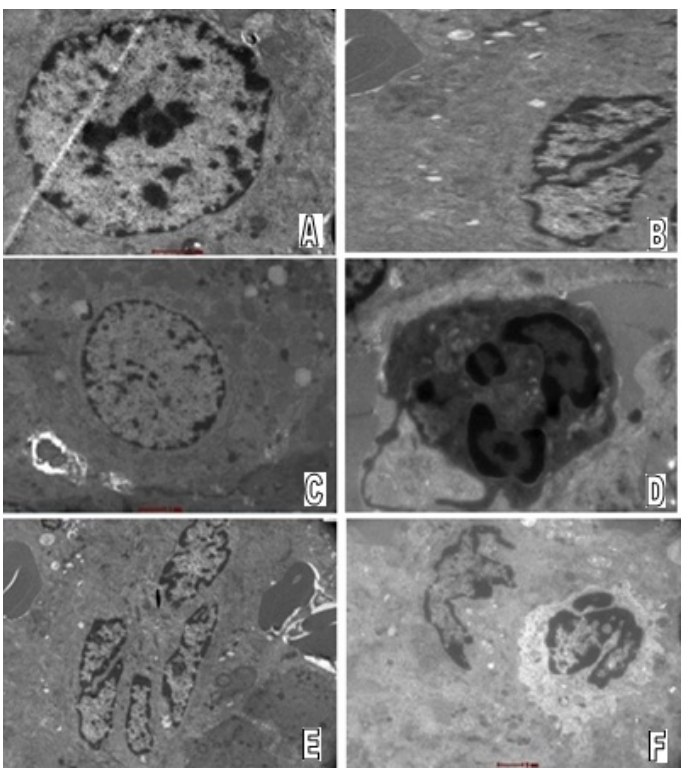

Figure 4: Effect of UMB, UMB + vitamin C on the ultra structural changes in the liver tissue of control and experimental rats

Ultra structural changes in control and experimental rats. Control (group 1) and drug control rats (group 3) showed normal liver architecture, nuclei and cytoplasm (4A and 4C). DEN induced rats (group 2) showed cells with multiple dysplastic nuclei close to each other with irregular shaped cytoplasm and defragmented nucleolar contents (4B). Pre-treated, post-treated and entire period treated rats (groups 4, 5 and 6) showed changes of apoptosis and liver cells with shrunken nucleus and condensed chromatin undergoing apoptosis (4D, 4E and 4F)

DISCUSSION

Histopathological examination of liver of control rats revealed normal liver architecture. However, DEN induced rats showed clear signs of severe hepatic injury manifested as areas with periportal and perivascular inflammatory infiltrates. Degenerative and regenerative cellular changes and proliferation of vascular channels were also noted. Drug control rats exhibited normal architecture, indicating the non-toxic nature of UMB and vitamin C. Pretreated and post treated rats showed moderate cancerous change, fatty change and hydropic degeneration. Rats treated for the entire period showed fewer neoplastically transformed cells and the hepatocytes maintaining near normal architecture and significant improvement in liver histopathology. Abnormal lipid synthesis or defective degradation of lipids has been implicated in pathological conditions like cancer[19]. The peroxidation of lipids in biological membranes and tissues causes the leakage of these lipids into circulation and consequently leads to hyperlipidemia. This has been reported to increase the risk of metastasis in cancers[20]. DEN induced liver injuries induce the accumulation of abnormal amounts of fat, predominantly triglycerides, in the parenchymal cells. In general, triglyceride accumulation can be thought of as resulting from an imbalance between the rate of synthesis and the rate of release of triglycerides by the parenchymal cells into the systemic circulation. The high concentration of triglycerides further results in increased levels of lysophosphatidic acid, which in turn is a potent stimulator of various cellular functions that are related to tumor development and metastasis. In the present study, the elevated levels of serum and tissue total cholesterol and triglycerides were observed in DEN induced cancer rats. This is in accordance with finding of Tang *et al*.[21] On the other hand, UMB and vitamin C treated rats showed decreased levels of total cholesterol and triglycerides. This might be attributed to the anti hyperlipidemic effect of UMB. Further, the synergistic action of UMB with vitamin C has been reported to reduce the cholesterol and triglycerides levels by regulating the membrane permeability and integrity. This significantly inhibits the hypertriglyceridemia, decreases the levels of lysophosphatidic acid and reduces the chances of malignancy. Several studies have revealed that membrane lipids mediate cellular responses to external stimuli and changes in phospholipids have been implicated in tumor development[22]. The decreased level of phospholipids and free fatty acids was seen in DEN induced rats. This is in accordance with finding of Ramakrishnan *et al*.[23] This decreased level of phospholipids might be due to increased phospholipid degradation. This could cause an alteration in structure and stability of the membranes, resulting in membrane dysfunction. The decrease in phospholipids might also be due to the decreased concentration of free fatty acids in tumor tissues which was brought back to near normal in pre, post and entire period treated rats. UMB and vitamin C supplementation to DEN treated rats (groups 4, 5 and 6) elevated the levels of serum and liver phospholipids which might be due to their decreased degradation. As a consequence, the deleterious effects of free radicals on the membrane were reduced, thereby preventing its dysfunction. UMB with vitamin C treatment significantly attenuated this decline in phospholipids and free fatty acids. Thus, UMB with vitamin C treatment appears to be an effective option against DEN induced HCC. Ultra-structural studies revealed normal nuclei and cytoplasm in the control and drug control rats. The normal architecture of drug control rats showed that the combination of UMB and vitamin C did not induce any changes in the intracellular morphology of liver cell. Also, this revealed the nontoxic nature of the combination at the given dosage. The rats induced with DEN showed the presence of multiple irregular shaped nuclei with irregular cytoplasm, which might be due to the excessive free radicals generation during DEN metabolism. Morphological changes of apoptosis (shrunken nucleus and condensed chromatin) were seen in the liver cells of pre treated and post treated and entire period treated rats. Thus, the results of the ultra structural studies undoubtedly confirmed that UMB and vitamin C have the ability to cause apoptosis in cancer cells.

CONCLUSION

In conclusion, combined action of UMB with vitamin C supplements showed potent hypolipidemic activity and are strong candidates as chemo preventive agents for the treatment of liver cancer.

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