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MODULATION OF MEMBRANE BOUND ATPASES AND METABOLIZING ENZYMES AGAINST N-NITOSODIETHYLAMINE (DEN) INDUCED PRIMARY LIVER CANCER IN WISTAR ALBINO RATS

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ABSTRACT

The present investigation was carried out to estimate the effect of bioactive compound limonin on various biological enzymes like Glycogen; Membrane bound ATPases, Carbohydrate metabolizing enzymes and Mitochondrial TCA cycle enzymes against *N*-nitrosodiethylamine (DEN) induced primary liver cancer in male Wistar albino rats. Liver cancer was induced by single intraperitonial injection of DEN (200 mg/kg). After 2 weeks of DEN administration, Phenobarbital (PB) was given to promote the cancer for up to 14 successive weeks. Limonin (50mg/kg/b.w/p.o) was given for 28 days to hepatocellular carcinoma bearing rats. After the experimental period, all the animals were sacrificed and serum and liver samples were collected for biochemical analysis. There was instability in the levels of biological enzymes when subjected to DEN induction. These altered enzyme levels were ameliorated significantly by administration of limonin at the concentration of 50mg/kg in drug-treated animals. This protective effect of limonin was associated with inhibition of LPO induced by DEN and to maintain these enzyme levels.

KEYWORS

Limonin, Hepatocellular carcinoma (HCC), Primary liver cancer, Membrane bound ATPases, N-nitrosodiethylamine (DEN), Mitochondrial TCA cycle enzymes.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide. The major etiologies and risk factors for HCC development are well-defined and hepatocarcinogenesis is developed by multiple steps process, which has been elucidated in recent years. HCC is a leading cause of cancer related death in the world [1]. N-nitrosodiethylamine (DEN) is a potent environmental hepatocarcinogen, shown to be metabolized to its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis [2, 3]. Investigational, scientific and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer. Diethylnitrosamine has been suggested to cause oxidative stress and cellular damage due to the enhanced formation reactive oxygen species [4, 5]. Limonoids are secondary metabolites in all citrus fruit tissues and unique highly oxygenated triterpenoid compounds are recognized as one of the most healthful components of the human diet [6]. Citrus limonoids appear in large amounts in citrus juice and citrus tissues as water soluble limonoid glucosides or in seeds as water insoluble limonoid aglycones [7]. Citrus limonoids have a number of biological properties, and structural characteristics that influence anticancer activity [8, 9], anti-HIV [10], and cholesterol lowering properties have been identified [11]. Animal studies have shown that citrus limonoids induce glutathione-S-transferase activity [12] and inhibit forestomach [13], oral [14], lung [15], skin [16], and colon [17] tumors in animals. Breast cancer cells line studies have shown limonoids to be potent inhibitors of the proliferation of estrogen receptor negative and estrogen receptor positive human breast cancer cells in culture [18].

Limonin is a bitter white crystalline substance found in citrus fruits, which is the bitter principle of citrus fruits. It is also identified as limonoate D-ring-lactone and limonoic acid di-delta-lactone. Limonin has been shown to possess anticarcinogenic properties in both *in vitro* and *in vivo* rodent models. In mice it was found that, among several limonioids have been identified, limonin, being a much better inducer of GST, was more active as an inhibitor of carcinogenesis [19]. In the present study, we investigated the effect of limonin on membrane ATPases, carbohydrate metabolizing and mitochondrial TCA cycle enzymes to assess the defensive role of limonin against DEN induced Phenobarbital (PB) promoted primary hepatocarcinogenesis in experimental rats.

MATERIALS AND METHODS

(i) Animals

Healthy adult male Wistar albino rats of weighing between 110±20g were used for the present study. They were obtained from the Central Animal House Facility, Dr.ALMPGIBMS, Taramani, University of Madras, Chennai (IAEC No: 07/012/08). The animals were kept in polypropylene cages and received standardized rat pellet and water *ad libitum.* All the procedures were done in compliance with the guidelines issued by the Institutional Animal Ethics Committee.

(ii) Chemicals

DEN was purchased from Sigma Chemical Company, St Louis, MO, U.S.A. All other Chemical including solvents used were of high purity and of analytical grade.

(iii) Experimental Design

In the present investigation rats were divided into four groups of six animals each. Group I: Control animals were administered with Dimethyl sulfoxide (DMSO) (1ml/kg/p.o). Group II: Animals received a single intra-peritoneal injection of DEN at a dose of 200mg/kg in normal saline. After two weeks, hepatocarcinogenesis was promoted by PB (0.05% PB). PB was administered to the animals through drinking water for 14 successive weeks [20]. Group III: After inducing primary liver cancer, animals were treated with limonin (50mg/kg/b.w/p.o) dissolved in DMSO (1ml) for a period of 28 successive days. Group IV: Animals received limonin (50mg/kg/b.w/p.o) dissolved in DMSO (1ml) for a period of 28 successive days. At the end of the experiment, all the animals were sacrificed by cervical decapitation. Blood and liver samples were collected for the estimate of various biochemical parameters.

(iv) Biochemical Estimations

(a)Estimation of Glycogen

Glycogen was estimated by the method of Morales *et al.,* (1973) [21].

(b) Estimation of Membrane bound ATPase Enzymes

Na^+K^+-ATPase was estimated by the method of Bonting, (1970) [22], The activity of Ca^{2+}-ATPase was assayed according to the method of Hjerten and Pan (1983) [23], Mg^{2+} -ATPase activity was assayed by the method of Ohinishi *et al.,* (1962) [24].

(c) Estimation of Carbohydrate metabolozing enzymes

Hexokinase was assayed by the method of Brandstrup *et al.,* (1957) [25], Phosphoglucoisomerase was assayed by the method of Horrrocks *et al.,* (1963) [26], Aldolase was estimated by the method of King (1965b), Glucose-6-phosphatease was assayed according to the method of King (1965b) [27], Fructose-1,6-diphosphatase was assayed by the method of Gancedo and Gancedo, (1971) [28].

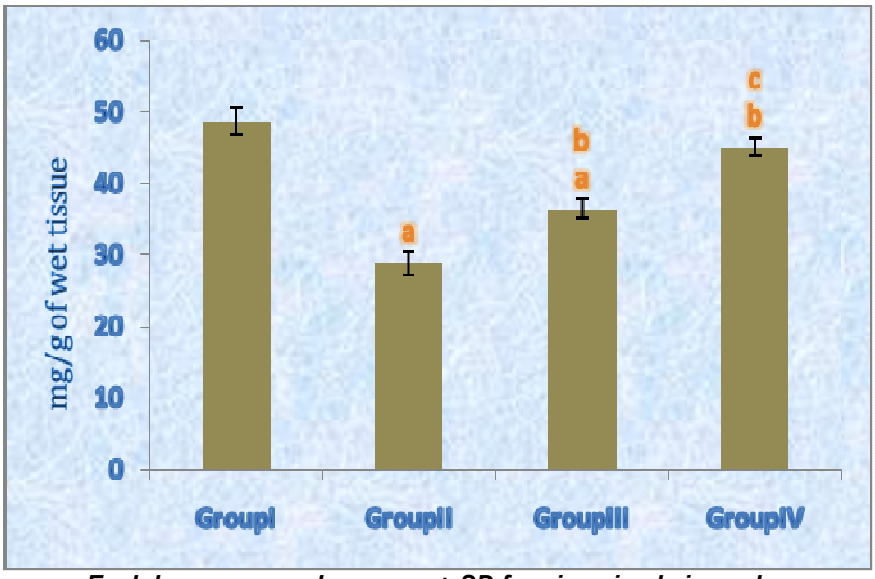
(d) Estimation of Mitochondrial TCA cycle enzymes

Estimation of Isocitrate dehydrogenase by the method of King (1965c) [29], Succinate dehydrogenase estimated by the method of Slater and Borner, 1952 [30]. Malate dehydrogenase assayed by the method of Mehler *et al.,* 1948 [31], α-ketoglutarate dehydrogenase by the method of Reed and Mukherjee, 1969 [32].

RESULTS

Figure 1 shows the levels of glycogen in liver of control and experimental animals. The liver glycogen level was found to be significantly decreased ($p<0.01$) in Group II DEN administered induced animals when compared to the control animals. Upon administration of limonin in Group III animals, the glycogen levels were brought back to near normal comparable to that of group II animals ($p<0.01$). Drug control Group IV animals did not show much variation when compared to control Group I animals.

Figure 1
The Levels of Glycogen in Liver of Control and Experimental Animals



Each bar expressed as mean ± SD for six animals in each group
a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV
c - Group III Vs Group IV, The significance at the level of p<0.01

Table 1 and 2 shows the activites of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase in erythrocyte membrane and liver respectively. In DEN induced group II animals, significant reduction in the levels of Na^+/K^+ ATPase, Mg^{2+} ATPase ($p<0.001$) and Ca^{2+} ATPase ($p<0.001$), were observed in erythrocyte membrane and liver when compared to control group I animals. These ATPases enzymes levels were increased in the limonin treated group III animals when compared with group II animals. No significant variations of all these enzymes level were observed in group IV limonin control animals when compared with group I animals.

Table 1

Levels of ATPase in erythrocyte membrane of control and experimental animals

Parameters (µ moles of inorganic phosphate liberated/mg protein/min)	Group I Control	Group II DEN	Group III DEN+ Limonin	Group IV Limonin
Na^+/K^+ ATPase	2.97±0.11	1.89±0.18a*	2.59±0.17b*	2.99±0.18c^{NS}
Ca^{2+} ATPase	2.17±0.17	1.65±0.20a*	2.23±0.12b*	2.15±0.11c^{NS}
Mg^{2+} ATPase	3.48±0.14	2.69±0.19a*	3.26±0.17b*	3.44±0.18c^{NS}

Each Value represents mean ±SD
****p<0.001, @p<0.01, #p<0.05, NS- Not significant, a- compared with group I; b-compared with group II; c-compared with group I***

Table 2
Levels of ATPase in liver of control and experimental animals

Parameters (µ moles of inorganic phosphate liberated/mg protein/min)	Group I Control	Group II DEN	Group III DEN+ Limonin	Group IV Limonin
Na^+/K^+ ATPase	0.42±0.08	0.29±0.09a*	0.40±0.02b*	0.39±0.02c^{NS}
Ca^{2+} ATPase	0.37±0.01	0.18±0.20a*	0.27±0.09b*	0.35±0.10c^{NS}
Mg^{2+} ATPase	0.30±0.02	0.25±0.001a*	0.26±0.008b*	0.31±0.01c^{NS}

Each Value represents mean ±SD
****p<0.001, @p<0.01, #p<0.05, NS- Not significant, a- compared with group I; b-compared with group II; c-compared with group I***

Table 3 shows the activities of the carbohydrate metabolizing enzymes in liver of control and experimental animals. In group II, DEN induced animals, the activities of enzymes such as hexokinase, phosphogluco-isomerase and aldolase were significantly increased and the gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1,6-diphosphatase enzymes were significantly decreased (p<0.001) when compared to group I control animals. Treatment with limonin in group III animals showed the carbohydrate metabolizing enzyme levels were significantly (p<0.001) brought back to near normal levels when compared with group II animals. No significant change in the activities of glycolytic and gluconeogenic enzymes were observed in limonin alone treated animals when compared with group I animals.

Table 3
Levels of Carbohydrate metabolizing enzymes in liver of control and experimental animals

Parameters	Group I Control	Group II DEN	Group III DEN+ Limonin	Group IV Limonin
Hexokinase (n moles of glucose-6-phosphate liberated/mg protein/min)	18.60±0.69	27.90±1.79a*	19.61±1.26b*	18.20±1.19c^{NS}
Phosphogluco-isomerase (n moles of fructose liberated/mg protein/min)	13.80±0.50	25.20±1.65a*	19.04±1.25b*	04.86±0.92c^{NS}
Aldolase (n moles of glyceraldehydes liberated/mg protein/min)	12.70±0.46	21.76±1.39a*	17.20±1.12b*	13.08±1.84c^{NS}
Glucose-6-phosphatase (n moles of inorganic phosphate liberated/mg protein/min)	12.41±0.46	8.41±0.53a*	10.80±0.66b*	11.76±1.84c^{NS}
Fructose-1,6-diphosphatase (n moles of inorganic phosphate liberated/mg protein/min)	18.02±0.69	3.08±0.86a*	5.40±0.99b*	17.20±1.12c^{NS}

Each Value represents mean ±SD
****p<0.001, @p<0.01, #p<0.05, NS- Not significant, a- compared with group I; b-compared with group II; c-compared with group I***

Table 4 shows the levels of ICDH, α-KGDH, SDH and MDH in liver of control and experimental animals. It was observed that there was significantly decrease (p<0.001) in the levels of TCA cycle enzymes in Group II DEN induced animals when compared to the

control group I animals. Upon administration of limonin in Group III animals, these levels were brought back to near normal comparable to that of group II animals ($p<0.05$). Drug control Group IV animals did not show much variation when compared to control Group I animals.

Table 4
Levels of Mitochondrial TCA cycle enzymes in liver of control and experimental animals

Parameters	Group I Control	Group II DEN	Group III DEN+ Limonin	Group IV Limonin
ICDH (n moles of α-ketoglutarate formed/mg protein/min)	6.54±1.20	4.99±0.25a*	5.19±2.16b*	6.50±1.13c^{NS}
SDH (n moles of succinate oxidized/mg protein/min)	4.10±3.22	2.55±0.82a*	3.51±1.31b*	4.16±1.90c^{NS}
MDH (n moles of NADH oxidized/mg protein/min)	6.70±0.28	3.66±1.41a*	4.95±1.24b*	6.81±1.50c^{NS}
α-KGDH (µ moles of potassium ferrocyanide liberated/mg protein/min)	1.85±0.25	0.65±0.18a*	1.12±1.21b*	1.90±1.10c^{NS}

Each Value represents mean ±SD
****p<0.001, @p<0.01, #p<0.05, NS- Not significant, a- compared with group I; b-compared with group II; c-compared with group I***

DISSCUSSION

Hepatocellular carcinoma (HCC) is one of the most widespread internal malignancies worldwide. Currently, there is no effective systemic chemotherapy for HCC, whereas alternative treatment strategies such as transcatheter arterial chemoembolization, percutaneous intratumoral ethanol injection and radiofrequency ablation are mainly for palliation and are applicable only to patients with tumours localized in the liver [33]. HCC is clearly a disease for which alternative therapeutic modalities must be developed. A thorough understanding of the pathogenesis of HCC thus holds the promise of finding an effective chemoprevention and treatment for this cancer [34].

Although traditionally cancer has been fought with the usual armamentarium of chemotherapy and high doses of directed radiation, lately there has been more attention devoted to combating cancer through nutritive means. In particular, a wide range of bioactive nutrients have been found useful in the physiological battle against cancer [35]. Ansari *et al.,* (1991) [36] have reported decreased levels of glycogen in 95% of experimental animals that were inflicted with chemical injury. The decreased glycogen levels in the present study may be due to the decrease in the number of hepatocytes. DEN induced cellular degradation and necrosis might have caused this decrease in the liver glycogen levels. Limonin administration increase the liver glycogen levels may be due to its anticarcinogeneic activity.

Adenosine tri phosphatases (ATPases) are vital enzyme for providing metabolic energy to the living processes. It regulates ion transport across cellular membrane, cellular volume, osmotic pressure and membrane permeability. It is an integral part of the membrane structure [37]. The membrane bound enzymes such as Na^+/K^+, Ca^{2+} and Mg^{2+} ATPase are responsible for the transport of sodium, potassium and calcium ions across the membrane. These lipids dependent enzymes have been implicated in the pathogenesis of liver injury [38]. In the present study, the level of Na^+/K^+ ATPases was found to decrease in erythrocyte membrane and liver of DEN induced HCC animals. Oxygen radicals have been reported to mediate inhibition of membrane bound ATPases by oxidizing the sulfphydryl groups in them [39].

The calcium pump Ca^{2+} ATPases in plasma membrane because of its high affinity has been proposed as the structure responsible for the maintenance of cytoplasmic calcium concentration at even submicoromolar level [40]. In the present investigation, it was observed that the level of Ca^{2+} ATPases was inhibited in erythrocyte membrane and liver in DEN administered animals. This may be due to the peroxidation of the membrane lipids by the carcinogen which initiates the loss of membrane integrity and enzyme activity [41]. It is well known that magnesium has a vital role in the maintenance of structure, metabolism and energestic of the cell. The synthesis of protein, nucleic acids and a number of other mitochondrial processes require magnesium. According to Frank *et al.,* (1999) [42] changes in the cytosolic Mg^{2+} concentration lead to a significant modification in cellular functions. Decreased levels of Mg^{2+} were observed in this present investigation may be attributed to increased LPO and membrane damaged by DEN. However limonin drug treated rats showed near normal levels. It reflects that limonin can protect the structural integrity and probably shield against the deleterious effect of lipid peroxidation.

It is well known that hepatic damage not only inflicts structural changes but also result in altered functionality of the liver, particularly the carbohydrate metabolism [43]. The present study showed an increase in the glycolytic enzymes such as hexokinase, aldolase and phosphoglucoisomerase and a significant decrease in glucose-6-phosphatase and fructose-1, 6-bisphosphatase in DEN treated animals. One of the strategies to prevent disease is the use of specific nutrients to protect tissues against toxic, carcinogenic injury and degenerative diseases [44, 45]. On limonin treatment, the enzymes level were reverted back to near normal levels due to the limonin activity of maintenance of glucose homeostasis.

Mitochondria, the energy reservoir of the cell is vital for producing energy for the sustenance of the cell. A damage to mitochondria leads to cell death [46]. The inner and outer membranes of mitochondria contain unsaturated lipids and they are susceptible to free radicals attack [47]. The mitochondrial damage due to DEN induced oxidative stress may affect the activities of TCA cycle enzymes such asisocitrate dehydrogenase (ICDH), Succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and α-ketoglutarate [48]. Increased free radical production thus compromises the ability to meet the energy demands of the cell by reducing the levels of mitochondrial TCA cycle enzymes. In the present investigation the levels of ICDH, SDH, MDH and α-KGDH were increased significantly due to limonin administration which may be due to its efficient in preserving the mitochondrial membrane integrity.

CONCLUSION

In conclusion, our investigation showed that limonin can modulate liver glycogen; membrane bound ATPases, carbohydrate metabolizing enzymes and mitochondrial TCA cycle enzymes, by reducing free radicals formed in DEN induced and Phenobarbital promoted primary hepatocarcinogenesis in experimental rats.

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