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RESEARCH ARTICLE

PROTECTION OF LIVER FROM *N*-NITROSODIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOGENESIS BY A NOVEL FLAVONOL–LUTEOLIN

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ABSTRACT

In this study, we analyzed the anticancer properties of luteolin in *N*-nitosodiethylamine induced group of rats. We found that the administration of luteolin (0.2mg) for 16 weeks to *N*-nitosodiethylamine induced rats provides protection against the oxidative stress caused by the carcinogen and thereby prevents hepatocellular carcinogenesis. On administration of the carcinogen, the level of lipid peroxidation elevated markedly, but it was found to be significantly reduced by luteolin administration. Besides, the antioxidant activities in serum were reduced in carcinogen administered animals, which were enhanced to normal level after luteolin treatment to *N*-nitrosodiethylamine induced group of rats and also this luteolin prevented the elevation of marker enzymes induced by *N*-nitrosodiethylamine. The bodyweight of the animals decreased and their relative liver weight increased significantly on *N*-nitrosodiethylamine administration when compared to control group. However, Treatment with luteolin significantly prevented the decrease of the body weight and increase in relative liver weight caused by DEN. In conclusion, these findings indicate that the compound prevent lipid peroxidation, hepatic cell damage and protect the antioxidant system in *N*-nitrosodiethylamine-induced hepatocellular carcinogenesis.

INTRODUCTION

N-Nitrosodiethylamine (DEN) is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water,cheddar cheese, cured and fried meals, occupational settings, cosmetics, agricultural chemicals and pharmaceutical agents (Brown, 1999, Sullivan *et al.,*1991, Reh and Fajen, 1996). It has been suggested that, on metabolic activation, it produces the pro-mutagenic products, *O* 6-ethyl deoxy guanosine and *O* 4 and *O* 6- ethyl deoxy thymidine in liver which are responsible for its carcinogenic effects (Verna *et al.,*1996). It is also reported that the generation of reactive oxygen species (ROS) by DEN causes carcinogenic effects. ROS are potentially dangerous by-products of cellular metabolism that have direct effect on cell development, growth and survival. Oxidative stress caused by ROS has been reported in membrane

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lipid peroxidation, DNA damage and mutagenesis associated with various stages of tumor formation process (Parola and Robino 2001). Hence the model of DEN-induced HCC is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis (Parola *et al.,* 2001). Human liver appears to metabolize nitrosamines in a manner similar to that of rodent liver and also exhibits considerable similarities with regard to morphology, genomic alterations and gene expression, despite their different disease etiologies (Feo *et al.,* 2000). Further, several flavonoids present naturally in food and also in plants, have been shown to modify critical reactions that cause inhibition of chemically induced hepatocarcinogenesis (Thorgeirsson *et al.,* 2002 and Bannasch and Nehrbass, 2001). Luteolin, 3′, 4′,5,7-tetrahydroxyflavone, belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects, and UV irradiation (Harborne JB, Williams, 2000). Evidence from cell culture, animal, and human population studies have suggested that flavonoids are also beneficial to human and animal health. Because of their abundance in foods, e.g., vegetables, fruits, and medicinal herbs, flavonoids are common nutrients that are antioxidants, estrogenic regulators, and antimicrobial agents (Birt *et al.,* 2001). It has been noticed that flavonoids may be a cancer preventive (Knekt *et al.,* 1997 and Neuhouse and Dietary, 2004). Flavonoids may block several points in the progression of carcinogenesis, including cell transformation, invasion, metastasis, and angiogenesis, through inhibiting kinases, reducing transcription factors, regulating cell cycle, and inducing apoptotic cell death. In the present study to analyze the anticancer properties of luteolin in DEN induced group of rats.

MATERIAL AND METHODS

Source of chemicals

Luteolin and DEN were purchased from Sigma Aldrich, USA and all other chemicals used were of analytical grade.

Animals

Male wistar albino rats of same age group and body weight 130-150g were selected for all the experiments. The animals were housed in polypropylene cages at an ambient temperature of 25–30ºC and 45–55% relative humidity with a 12 h each of dark and light cycle. Rats were fed pellet diet and water *ad libitum.* The study was approved by the Institutional Ethical Committee.

Experimental protocol

The experimental animals were divided into three groups, each group comprising of six animals for a study period of 16 weeks as follows: group 1, normal control rats fed with standard diet and pure drinking water. Group 2 rats were induced with DEN (100 mg/kg bodyweight once a week for three weeks. Ip). In group 3 rats received 0.2 mg of luteolin was administered to DEN induced group of rats for 16 weeks. At the end of the experimental period, the rats were sacrificed by cervical dislocation. The blood was collected for further biochemical analysis. All the animal experiments were duly approved by the Institutional Animal Ethics Committee (743/03/abc/ CPCSEA dt 3.3.03) Guidelines.

Biochemical assay

Aspartate transaminase, Alanine transaminase, Acid phosphatase, alkaline phosphatase albumin, globulin and α feto protein (AFP) were estimated by using commercially available kits according to the manufacturer's instruction.(AGGAPPE Diagnostic, Kerala, Ensure Biotech Pvt, Hyderabad, India and (ELISA KIT) UBI, MAGIWELL (USA).

Protein determination

Rat liver organs were homogenized in 10 times their weight of phosphate buffer, the homogenate centrifuged for 15 min at 4ºC and the supernatants used for measurement of protein estimation. Protein content was determined by the method of Bradford (Bradford, 1976).

Antioxidant and lipidperoxidation

The activities of enzymatic antioxidants such as SOD (Kakkar *et al.,* 1984), Catalase (Sinha, 1982), were assayed in serum of control and experimental

group of rats. Further, the Levels of lipid peroxides (Berton *et al.,* 1998) were determined in the serum of control and experimental groups of rats.

RESULTS

The anticancer efficiency of luteolin against DEN induced hepatocellular cancer was analyzed in male Wistar albino rats. Fig.1 shows that the body weight reduced significantly ($p < 0.05$) in DEN-induced animals compared to control group of rats whereas it was normalized by rats treated with luteolin. Fig. 2 indicates that administration of DEN to animals caused a significant ($p < 0.05$) increase in liver weight due to appearance of liver nodules. However, rats administered with luteolin the liver weight were reversed to normal weight.

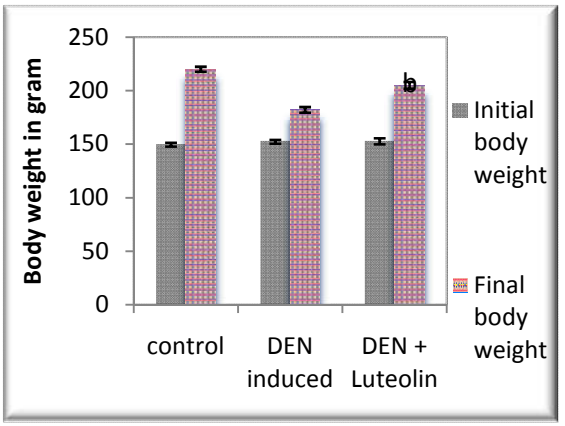

Fig. 1. Body weight of control and experimental groups of rats. Values are expressed as mean±S.D. (n = 6). [a] P<0.05 compare with control groups of rats, [b] P<0.05 compare with DEN induced groups rats

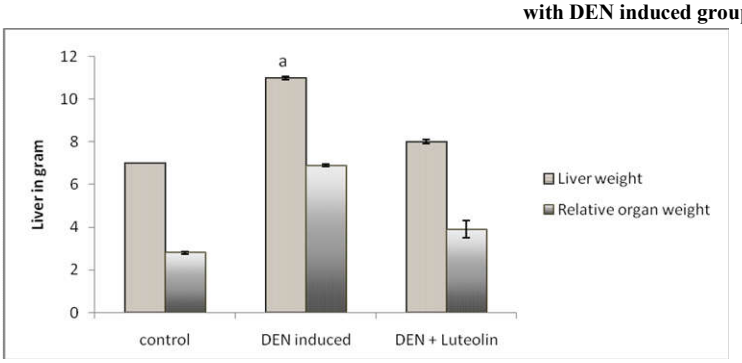

Fig. 2. Liver weight and relative liver weight of control and experimental groups of rats. Values are expressed as mean±S.D. (n = 6). [a] P<0.05 compare with control groups of rats, [b] P<0.05 compare with DEN induced groups rats

Table 1. Effect of luteolin on serum total protein, albumin and globulin

Groups	Protein(gm %/dl)	albumin(gm/dl)	Globulin(gm/dl)
Control	6.1±0.04	0.98±0.26	2.95±0.52
DEN induced rats	3.5±0.01[a]	1.85±0.31[a]	1.90±0.23[a]
DEN+Luteolin	5.8±0.26[b]	1.03±0.42[b]	2.51± 0.21[b]

Table 2. Effect of luteolin on serum pathophysiological enzymes

Groups	AST(U/L)	ALT(U/L)	ALP(U/L)	LDH(U/L)
Control	60±2.3	52±2.6	160±2.8	190±4.1
DEN induced	140±2.6[a]	132±1.2[a]	210±1.4[a]	240±4.3[a]
DEN+Luteolin	90±3.1[b]	93±1.7[b]	180±2.3[b]	210±3.8[b]

[a] Comparisons are made with group 1 (control).
[b]. Comparisons are made with group 2 (DEN-induced).

Table 3. The production of lipid peroxides in serum carcinogen-administered group of rats

Groups	MDA(nm/mg of protein)	Catalase (U/mg of protein)	SOD (U/mg of protein)
Control	2.13±0.41	17.54±.0.42	415.06±0.43
DEN induced	3.85±0.24[a]	10.13±0.42[a]	356.64±0.42[a]
DEN+Luteolin	2.10±0.13 [b]	14.42±0.14[b]	396.01±0.12[b]

Values are expressed as mean±S.D. (n = 6). [a] P<0.05 compare with control groups of rats, [b] P<0.05 compare with DEN induced groups of rats

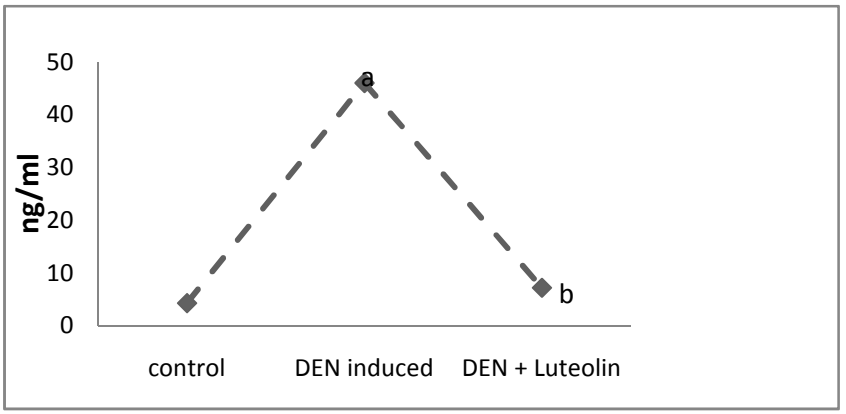

Fig. 3. Values are expressed as mean±S.D. (n = 6). Statistical significance p < 0.05. Activity is expressed as ng/ml for α feto protein

[a] Comparisons are made with group 1 (control).

[b]. Comparisons are made with group 2 (DEN-induced).

content of protein in control, cancer induced, and treated groups are presented in Table 1. The serum total protein content and globulin decreased in DEN-induced animals whereas albumin level was increased as compared to normal control. However, after treatment with luteolin the altered level were normalized. With regard to serum phathophysiological enzymes, effects of luteolin on AST, ALT, ALP and LDH (Table 2). DEN-induced animals significantly increased ($p < 0.05$) the level of SGOT, SGPT, ALP and LDH as compared to normal control. By contrast, at a dose of 0.2 mg/kg of luteolin administration significantly reversed the altered level of the phathophysiological enzymes in serum as compared to the DEN-induced rats. Table 3 shows the production of lipid peroxides in serum carcinogen-administered groups of animals. A significant increase in LPO level ($p < 0.05$) did not occur at treatment with Luteolin. Besides, the activity of catalase and SOD was significantly lower in carcinogen-administered group of animals when compared with control groups of rats, Nevertheless, rats administered with Luteolin significant enhancement were observed when compared with in carcinogen-administered group of rats. The Fig. 3 depicts the level of the tumor markers protein α feto protein (AFP). Their levels were found to be elevated significantly ($p < 0.05$) in DEN-induced animals where as their levels were significantly lower on treatment with luteolin.

DISCUSSION

Liver damage caused by DEN generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities (Plaa and Hewitt, 1989). Serum SGOT, SGPT, ALP and ACP are representative of liver function; their increased levels are indicators of liver damage. The elevation of ALT activity is

repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST (Plaa and Hewitt, 1989). Increase in ALP reflects the pathological alteration in biliary flow.In the present study, treatment with luteolin attenuated the increased activities of these enzymes were normalized. It suggested that the luteolin assist in parenchymal cell regeneration in liver, thus protecting membrane integrity, thereby decreasing enzyme leakage.

Reactive oxygen species degrade polyunsaturated lipids to produce the malondialdehyde. This compounds is a reactive aldehyde and is one of the many reactive electrophiles species that cause toxic stress in cells and form covalent protein adducts which are termed to as advanced lipoxidation end products in analogy to advanced glycation end products The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism. Lipid peroxidation is one of the major mechanisms of cellular injury caused by free radicals(Esterbauer and Chesseman, 1990). Administration of DEN has been reported to generate lipid peroxidation products like MDA and 4-hydroxy nonenal that may interact with various molecules leading to oxidative stress and carcinogenesis (Hietanen, *et al.,* 1987).The level of LPO increases with the administration of DEN during hepatocarcinogenesis. SOD is an important defense enzyme which catalyses the dismutation of superoxide radicals to hydrogen peroxide thereby reducing the likelihood of superoxide anion interacting with NO to form reactive peroxynitrite (Maritim *et al.,* 2003). Hydrogen peroxide is successively metabolized into water and non reactive oxygen species by the activities of catalase and GPx (Matés and Sánchez-Jiménez, 1999). Catalase a tetrameric enzyme which is that decomposes the hydrogen peroxide into the harmless products such as water and molecular oxygen. Hydrogen peroxide toxicity is enhanced to the formation of reactive hydroxyl radical on capture of an electron from Fe (II) or Cu (I). The hydroxyl radical reacts instantly with all cellular components resulting in the modifications of protein and nucleic acids. Catalase is one of the most competent enzymes so that it cannot be saturated by hydrogen peroxide at any concentration (Lledías *et al.,* 1998). Decreases in the activities of SOD, CAT are observed in tumor cells. The luteolin that can scavenge excessive free radicals in the body are suggested to hinder the process of carcinogenesis. Such studies support our findings that activities of the enzymic antioxidants are reverted to near normal in Luteolin treated animals and hence prevent the initiation of carcinogenesis by DEN. The protection offered by luteolin to the enzymatic antioxidant system may be explained by the increase in the level of these antioxidants probably due to the direct reaction of luteolin with ROS. Luteolin may also protect the membrane and antioxidants from ROS. α Feto protein (AFP) an oncofetal serum protein, progressively lost during development, such that it is virtually absent from the healthy adult (Sell *et al.,* 1983). It has long been recognized that exposure of rats to certain carcinogens like DEN causes an elevation of circulating AFP levels. This corroborates with the results showing the significant rise in levels of AFP obtained in DEN-induced animals (Becker and Sell, 1979) that were found to be reduced in luteolin treated animals.

Our results highlight the ability of luteolin to change the levels of LPO and significantly increase the endogenous antioxidant defense mechanisms in DEN induced hepatocellular carcinogenesis. Our results also show that the significant increase in the levels of serum markers and tumor markers was prevented by luteolin treatment. From the results obtained, we suggest that luteolin may be developed as an effective chemotherapeutic agent. Further studies are underway to elucidate the molecular mechanisms involved to prove luteolin efficacy as an anti-hepatocarcinoma agent.

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