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Therapeutic Efficacy of Allyl Isothiocyanate Evaluated on N-Nitrosodiethylamine/Phenobarbitol induced Hepatocarcinogenesis in Wistar Rats

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Abstract: N-nitrosodiethylamine (NDEA) is a potential carcinogenic agent that induces liver cancer. To evaluate the chemotherapeutic effect of Allyl isothiocyanate in the experimental model, Wistar male rats were administered single dose of intra peritoneal (IP) injection of NDEA. Two weeks after administration of NDEA, Phenobarbital at the concentration of 0.05% was incorporated in rat chow for up to 14 successive weeks to promote liver cancer. Allyl isothiocyanate (AITC) (2mg/kg body weight) in addition with 0.5ml of corn oil was given orally on a daily basis. At the end of this experimental period, the rats were sacrificed and the blood samples were taken for biochemical studies. The levels of the marker enzymes for liver function were measured in serum. The results of the biochemical studies showed that NDEA administration followed by phenobarbital induces macro and microscopic liver tumors that increase the levels of marker enzymes and decreases the level of antioxidant in the serum in addition to loss of body weight. Conclusively, the administration of AITC as therapeutic treatment for hepatocarcinoma has significantly reduced the tumor development and counteracted all the biochemical effects induced by NDEA.

Keywords: Hepatocarcinogenesis, Allyl isothiocyanate, Antioxidants, Marker enzymes, Alfa fetoprotein.

1. Introduction

N-nitrosodiethylamine (NDEA) is a dialkyl nitrosamine, belongs to the group of N-nitrosamines causing a wide range of tumors in all animal species and suspected to be health hazardous to man and used as a carcinogenic agent to induce liver cancer in animal models. Exposure of man to N-nitrosamines occurs through consumption of products such as salt preserved food, tobacco, cosmetics, pharmaceutical and agricultural chemicals. During the metabolism of NDEA, the formation of reactive oxygen species (ROS) results in oxidative stress, which may be one of the key factors in the etiology of liver cancer [1, 2]. Isothiocyanates (ITCs) are a family of compounds derived exclusively from plants, also marine sponges and fungi have been reported to produce a few ITCs [3]. Recent evidence suggests that isothiocyanates might be involved in various but much less characterized mechanisms such as induction of cell-cycle arrest and apoptosis in human cancer cells *in vitro*. Examples that are particularly rich in various ITCs which include popular crucifers' mustard and horseradish for allyl-ITC (AITC) [4], watercress for phenethyl-ITC (PEITC) [5], broccoli and broccoli sprouts for sulforaphane (SF) [6]. AITC extracted from mustard seeds contains both antiangiogenic and proapoptotic activities [7]. To date, the most important known biological activity of ITCs is their ability to inhibit cancer development. There is convincing evidence that certain natural ITCs, such as AITC, benzyl ITC (BITC), PEITC and SF as well as a number

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of synthetic analogs are effective inhibitors of chemically induced tumors in one or more organ sites [3]. In a previous investigation, AITC significantly inhibited the formation of gastric lesions induced by 0.6M HCl, 1% ammonia, aspirin and ethanol. Allyl isothiocyanate also significantly inhibited the lesions induced by indomethacin, but a dose-dependent inhibition was not observed. Other synthetic isothiocyanate compounds also substantially inhibited the formation of lesions generated by ethanol, but their effects on indomethacin-induced gastric lesions were weaker than those on ethanol-induced gastric lesions similar to allyl isothiocyanate [8]. We carried out this study to investigate the effects of Allyl isothiocyanate in preventing the tumor development and oxidative damage caused by NDEA induced hepatic cancer in experimental model rats.

2. Materials and Methods

2.1 Animals

Male adult albino rats of Wister strain weighing between 180 ± 30 g were procured from the Central Animal House Facility, Dr. A.L.M. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai, India. The animals were kept in polypropylene cages and fed with standard diet and water ad libitum. Guidelines for experiments on animals as mentioned by the Ministry of Social Justice and Empowerment of India were followed (IAEC: 07/013/08).

2.2 Chemicals

N-nitrosodiethylamine and Allyl isothiocyanate were purchased from Sigma Chemical Company, St Louis, MO, USA. All the other chemicals and solvents used in the study were of analytical grade.

2.3 Experimental design

The rats were divided into four groups of six animals in each group and the dose regimen as given below. Group, I served as control animals were given only 1ml of corn oil for 28 days. Group II animals and Group III were given single intra peritoneal (IP) injection of NDEA at a dose concentration of 200 mg/kg body weight mixed in saline to induce liver cancer. Two weeks after administration of NDEA, 0.05% of PB was incorporated in rat chow for these groups up to 14 successive weeks to promote the liver cancer. Only Group III liver cancer bearing animals were treated orally with AITC (dissolved in 1ml of corn oil) at a concentration of 2 mg/kg body weight for 28 days. Group IV animals were treated orally with AITC only and served as drug control.

2.4 Biochemical assays

As marker enzymes for liver function in serum, the activity of aspartate transaminase (AST) and alanine transaminase (ALT) King (1965a) [9]; Alkaline Phosphatase (ALP) Bergmayer (1963), as described by Balasubramanian et al., (1983) [10]; Lactate Dehydrogenase (LDH) King (1965b) [11], were assessed. Estimation of α -Fetoprotein (AFP) was measured quantitatively by the method of solid phase enzyme linked immunosorbent assay (ELISA). In addition, the activity of superoxide dismutase (SOD) Marklund and Marklund (1974) [12]; catalase (CAT) Sinha (1972) [13] and glutathione peroxidase (GPx) Rotruck et al., (1973) [14], were also assessed in serum. All assay procedures were carried out according to the standard method as prescribed.

2.5 Statistical analysis

Data are presented as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey's multiple comparison methods was used to compare the means of different groups by using SPSS 7.5 student versions. Comparisons were made between group I with II and IV, and group III with II, and group IV with the group I for animal studies.

3. Results and Discussion

N-nitrosodiethylamine is present in major dietary sources like cured meats, salami, millet flour and dried cuttlefish [1, 2]. NDEA is metabolized primarily in the liver by cytochrome P450 to ethyl-acetoxyethyl nitrosamine. This intermediate can be conjugated by the phase II enzymes to a nontoxic compound or it can produce ethyldiazonium ion that directly ethylates cellular macromolecules [15]. Metabolic activation of NDEA by cytochrome P450 enzymes is responsible for its cytotoxic, mutagenic and carcinogenic effects. Instrumental to them is the increase in ROS production caused by NDEA [16]. Similar to the previous studies, NDEA brought about a significant impairment in body growth ($p < 0.001$) which was partially counteracted by the administration of Allyl isothiocyanate. Table 1 summarizes the effect of Allyl isothiocyanate on carcinogen induced changes in the body and liver weight. After NDEA dosage, treatment with allyl isothiocyanate administration augmented liver weight significantly ($p < 0.05$), an effect of NDEA suppressed.

Table 1.

Weight (g)	Group I	Group II	Group III	Group IV
Body	153.83 ± 10.80	126.66 ± 10.80 a*	154.33 ± 10.80 b*	152..66 ± 11.90 c^{NS}
Liver	6.59 ± 0.45	7.84 ± 0.45 $a^{\#}$	7.30 ± 0.34 b*	6.54 ± 0.17 c^{NS}

Each value represents mean + SD for six animals; a – Group I compared with Group II & IV; b – Group III compared with Group II; c- Group IV compared with Group I; NS – nonsignificant; *- $p < 0.001$, @ - $p < 0.01$, # $p < 0.05$

Aspartate transaminase and ALT activities in serum are indexes of liver damage [17]. In our study, elevated activities of AST and ALT, ALP and LDH were observed in the serum of NDEA-treated group of animals. Similar results were obtained in earlier studies [18] which are indicative of NDEA-induced hepatic damage and subsequent leakage of these enzymes into the systemic circulation. An analysis of five parameters indicating liver functional damage was represented in the serum as AST ($p < 0.001$), ALT ($p < 0.001$), ALP ($p < 0.001$) and LDH ($p < 0.001$) activities (Fig. 1).

These results instigate that the effect of allyl isothiocyanate to NDEA-treated animals have significantly decreased the level of AST, ALT, ALP and LDH. Another marker of hepatic damage is α-feto protein which is highly expressed in hepatocytes and activated early in hepatogenesis and expressed postnatally. This marker protein can be reactivated during liver regeneration and in hepatocellular carcinoma [19]. The result of the α- fetoprotein levels exhibited comparable changes ($p < 0.001$) after experimental manipulation (Fig. 2). NDEA treatment augmented the level of α-fetoprotein while Allyl isothiocyanate partially counteracted the effect of it. The level of α-fetoprotein attributes a significant cytoprotective effect on the hepatocytes and the exact mechanisms through which the cytoprotective effect is exerted remains to be defined. Reactive oxygen species (ROS) generation is a major factor involved in all steps of carcinogenesis, i.e. initiation, promotion, and progression [20]. Oxidant–antioxidant balance impacts the rate of cell proliferation, and tumor cells generally display low levels of lipid peroxidation which, in turn, can stimulate cell division and promote tumor growth [21]. It has been reported that mild oxidative stress stimulates cell growth while excess oxidative stress inhibits it [22]. Glutathione serves numerous important functions including antioxidant defense, maintenance of intracellular redox state and modulation of cell proliferation. Similarly, GPx catalyzes the reduction of peroxides and GST is a critical detoxification enzyme that conjugates functionalized P450 metabolites with GSH. These metabolizing enzymes are over expressed during active cell proliferation [23].

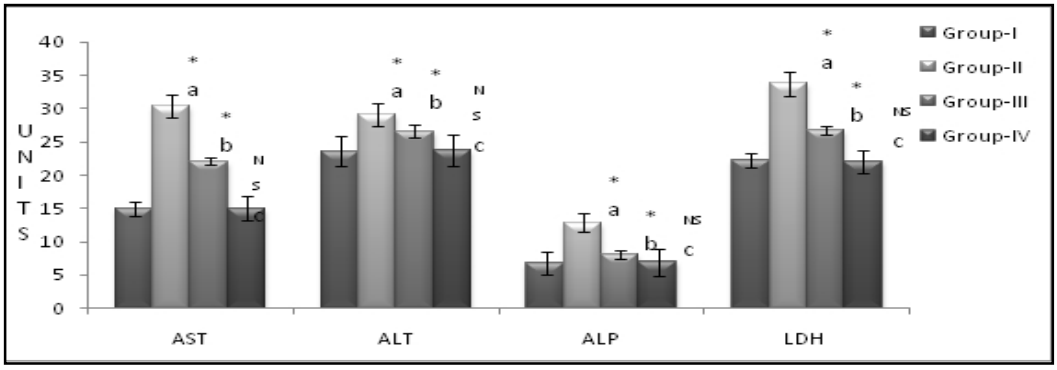

Figure 1.

Each value represents mean ± SD for six animals; Units – AST - μ moles of pyruvate liberated / mg protein/min; ALT- μ moles of pyruvate liberated / mg Protein / MIN; ALP - μ moles of p-nitrophenol liberated / mg protein/min; LDH - μ moles of pyurvate liberated / mg protein / min; a – Group I compared with Group II & IV; b – Group III compared with Group II; NS – nonsignificant; * - $p < 0.001$, @ - $p < 0.01$, # $p < 0.05$;

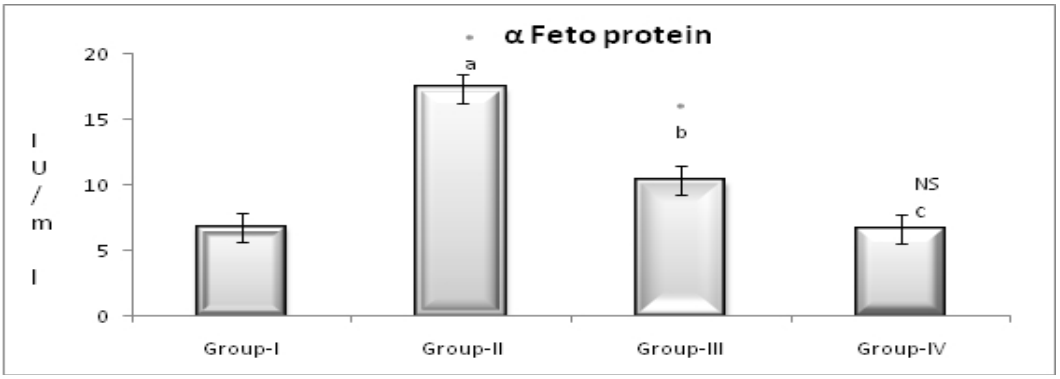

Figure 2.

Each value represents mean + SD for six animals; a – Group I compared with Group II & IV; b – Group III compared with Group II; c^{NS} – nonsignificant; * - $p < 0.001$, @ - $p < 0.01$, # $p < 0.05$;

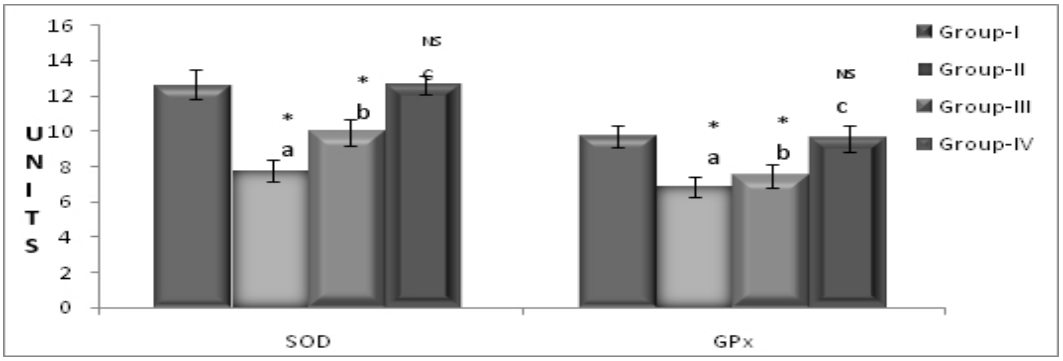

Figure 3.

Each value represents mean + SD for six animals; SOD – Units/mg protein; GPx – mg of GSH utilized/mg protein/min; a – Group I compared with Group II & IV; b – Group III compared with Group II; c^{NS} – nonsignificant; * - $p < 0.001$, @ - $p < 0.01$, # $p < 0.05$;

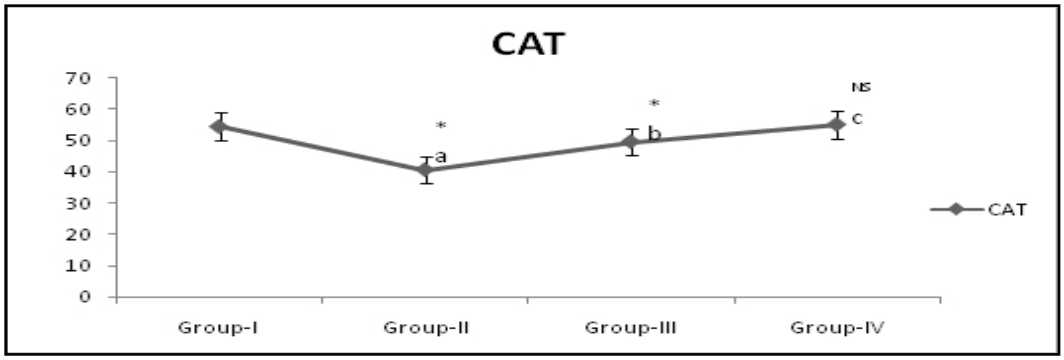

Figure 4.

Each value represents mean + SD for six animals; a – Group I compared with Group II & IV; b – Group III compared with Group II; c^{NS} – nonsignificant; * - $p < 0.001$, @ - $p < 0.01$, # $p < 0.05$;

Superoxide dismutase and CAT constitute a mutually protective set of enzymes against ROS. The activities of antioxidant enzymes especially SOD and its manganese-containing isoform are reduced when malignancies are present [24, 25]. These previous attributions were well correlated with our result instigating that the activities of serum CAT, SOD, GST and GPx in the serum decreased. The levels of these lessen during carcinogenesis ($p < 0.001$) (Figs. 3 and 4).

Carcinogenic substances inhibit SOD and CAT activities by a direct mechanism while anti carcinogens increase the activities of these detoxifying enzymes. This appears to be the case in the previous report for Allyl isothiocyanate which increases tissue SOD mRNA levels and also the activity of CAT [26]. This finding was well corroborated with our result implies that the effect was counteracted by Allyl isothiocyanate ($p < 0.001$) (Fig. 4).

In blood, depleted levels of antioxidants were observed in NDEA treated rats possibly as a consequence of NDEA-induced ROS generation [27]. Furthermore, tumor cells also sequester antioxidants from circulation to promote tumor growth [28]. The administration of Allyl isothiocyanate reverted NDEA induced alterations of enzyme activities in serum. Collectively, the data indicate one of the mechanisms by which Allyl isothiocyanate may exert its chemopreventive role is the modulation of lipid peroxidation and antioxidants in both the target organ and the circulation.

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