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Hepatoprotective and Antioxidant Effect of Vitamin C and E Against Some Common Non-steroidal Antiinflammatory Drugs Induced Hepatic Damage in Rats

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Non-steroidal antiinflammatory drugs (NSAIDs) are among the most frequently prescribed drugs worldwide. Hepatotoxicity is the most serious adverse effects of aceclofenac and diclofenac sodium. This study was designed to evaluate the hepatoprotective activity of vitamin C and E against NSAIDs induced acute liver injury in rats and to determine its mechanism of action. Administration of vitamin C and E (200 mg/kg/d) as a food supplement for 28 d significantly reduced the elevated relative values of liver weight, serum transaminases (alanine aminotransferase and aspertate aminotransferase), alkaline phosphatase, blood urea nitrogen, bilirubin (total and direct), total cholesterol and the hepatic morphological changes induced by aceclofenac (120 mg/kg/d) and diclofenac (120 mg/kg/d) in rats. However, simultaneous treatment with vitamin C and E as a food supplement (200 mg/kg/d) significantly attenuated aceclofenac and diclofenac induced hepatotoxicity. Histopathological damage of liver and the number of apoptotic hepatocytes were also significantly ameliorated by vitamin C and E treatment. It is therefore, suggested that combination of vitamins C and E had significant hepatoprotective activity and its mechanism is related at least in part, to its antioxidant properties.

Key Words: Aceclofenac, Diclofenac, Vitamin C and E, Hepatotoxicity, Antioxidant.

INTRODUCTION

Non-steroidal antiinflammatory drugs (NSAIDs) such as aceclofenac and diclofenac sodium are extensively used as analgesics and antiinflammatory agents and produce their therapeutic effects through the inhibition of prostaglandin synthesis[1,2]. Aceclofenac and diclofenac are oral non-steroidal antiinflammatory drugs (NSAIDs) belongs to the aryl-acetic acid class, are commonly used NSAID in several countries. These two drugs are effective in the treatment of painful inflammatory diseases and have been used to treat more than 75 million people worldwide[3]. Chronic usage of aceclofenac and diclofenac damages gastrointestinal mucosa by irritant action, causing alteration in mucosal permeability and/or suppression of prostaglandin synthesis.

The use of oral non-steroidal antiinflammatory drugs is associated with upper gastrointestinal complications, particularly perforated and bleeding peptic ulcer[4]. Nearly all of the NSAIDs have been implicated in causing liver injury and tend to be hepatocellular in nature. The mechanism is thought to be immunological idiosyncrasy[5,6]. The exact mechanism is not known but it is probably related to the decrease in the fatty acid entering the cell or release from the cell.

A group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body's natural defenses against free radicals (*e.g.* antioxidants) are inhibited by aceclofenac and diclofenac consumption, leading to increased liver damage.

Ascorbic acid (vitamin C) may protect lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals at early stage. The antioxidant function of ascorbic acid is related to its reversible oxidation and reduction characteristics. Thus, ascorbic acid may partially prevent certain types of hepatic cellular damage[8,9].

Vitamin E is a fat-soluble vitamin that exists in eight different forms. Each form has its own biological activity, which is the measure of potency or functional use in the body[10]. Vitamin E acts to protect the cells against the effects of free radicals, which are potentially damaging by-products of energy metabolism[11,12].

The aim of this study was to investigate the possible hepatoprotective effects of vitamin C and E on the serum hepatospecific markers and free radical damage of liver caused by aceclofenac and diclofenac sodium in rats.

EXPERIMENTAL

This study was carried out on 30 male rats (Wistar albino). The rats weighted 110-120 g and were the same age (3.0-3.5 months old). The experimental animals were randomly divided into five groups of six animals and were housed in cages at 22-25 ºC in a dark-light cycle. During the study, the animals were fed with standard laboratory diet (Hindustan Liver) and received water and pellet food *ad libitum*. All procedures described were reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

Experimental design: The rats were divided into the following groups each containing 6 rats (n = 6).

Group I: Control rats which were fed normal diet and water.

Group II: Aceclofenac treated rats: 120 mg aceclofenac/kg body weight/day for 4 weeks.

Group III: Aceclofenac + vitamin treated rats: 120 mg aceclofenac/kg body weight/day + 200 m g vitamin C + 200 mg vitamin E/kg body weight/day as a food supplement for 4 weeks.

Group IV: Diclofenac treated rats: 120 mg diclofenac/kg body weight/day for 4 weeks.

Group V: Diclofenac + vitamin treated rats: 120 mg diclofenac/kg body weight/day + 200 mg vitamin C + 200 mg vitamin E/kg body weight/day as a food supplement for 4 weeks.

Rats were monitored daily for general health and body weight was recorded every week during the entire period of the study. At the end of the experimental period, food was withheld overnight and rats were sacrificed by decapitation.

Drugs and chemicals: Aceclofenac and diclofenac sodium were obtained from Dey's Medical Stores (Mfg.) Ltd., India. Vitamin C and E were obtained from sigma UK. All biochemical kits were obtained from Merck, Germany. TBA, TCA and HCl were obtained from Ranbaxy Pvt. Ltd., India. Chemicals for SOD, GSH and Catalase were obtained from sigma UK. All other reagents used for the experiments were of analytical grade.

Serum and tissue preparations: Immediately after sacrifice blood was collected by heart puncture. The liver was removed and washed with ice-cold saline. The collected blood was kept at room temperature for 0.5 h to allow clotting and serum was then separated by centrifugation at 1500 g for 10 min. A small portion of the liver tissue was then cut into pieces and homogenized with 3 volumes (w/v) of the appropriate buffer using a Potter-Elvehjam homogenizer with a Teflon pestle and centrifuged at 12000 g for 20 min at 4 ºC. The supernatant was used for the biochemical estimation.

Biochemical analysis: Serum alanine amino transferase (ALT), serum aspertate amino transferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), Bilirubin (total and direct) and serum cholesterol were determined using commercial enzyme assay kits (Merck, Germany) according to the manufacturer's instructions.

Antioxidant enzyme assay: SOD was assayed using the method of Kakkar *et al.*[13] based on 50 % inhibition of the formation of NADH phenazine methosulfate-nitroblue tetrazolium (NBT) formazan at 520 nm. One unit of the enzyme required for 50 % inhibition of NBT reduction per minute per mg protein.

The activity of CAT was determined by the method of Sinha[14]. The values of CAT activity are express as μmol of H_2O_2 utilized per minute per mg protein.

Reduced glutathione (GSH) was determined by the method of Ellman[15]. The values are expressed as mmol (mg tissue)$^{-1}$. GPx activity was assayed by the method of Rotruck *et al.*[16], the values are expressed as μmol of GSH utilized per minute per mg protein.

Determination of total protein: Total protein was determined according to the method of Lowry *et al.*[17].

Histopathological analysis: Portions of the liver were then fixed in buffered form formalin, processed through graded alcohol and xylene and embedded in paraffin wax following the standard microtechnique given by Galighar and Kozloff[18]. 5-6 μ Sections were made at multiple levels and stained routinely with hematoxylin and eosin. Mounted slides were examined and photographed under a light microscope.

Statistical analysis: Data were analyzed by two-way analysis of variance and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $p < 0.05$. All statistical analyses were made using SPSS 11.0 software package (SPSS, Tokyo, Japan).

RESULTS AND DISCUSSION

Effect of vitamin C and E on liver weight and liver function: The relative liver weight was significantly augmented after treatment with aceclofenac and diclofenac separately compared with the normal control group. In contrast, pre-treatment with vitamin C and E (200 mg/kg/d as a food supplement) significantly reduced the relative liver weights compared with rats that received aceclofenac and diclofenac. Similarly the NSAIDs treated groups had elevated serum ALT, AST, ALP, BUN, bilirubin, total cholesterol levels, demonstrating marked liver damage. Pretreatment with vitamin C and E attenuated the NSAIDs induced increased hepatic enzyme activity (Table-1).

Effect of vitamin C and E on hepatic liver peroxidation: The level of lipid peroxidation in liver tissues, as measured by the concentration of hepatic TBARS, was significantly increased in rats that received aceclofenac and diclofenac treatment separately compared with the normal control group. Administration of vitamin C and E for 28 d led to a significant decrease in the degree of lipid peroxidation (Table-2).

Effect of vitamin C and E on the activity of hepatic antioxidant enzymes: The activity of antoxident enzymes, including SOD, CAT and GSH were significantly inhibited in the liver tissue of rats treated with NSAIDs. Pretreatment with vitamin C and E markedly increased the levels of SOD, CAT and GSH (Table-2).

Liver histopathology: No histopathological abnormalities were observed in normal control rats. The hepatic parenchyma appeared normal and hepatocytes were arranged around the central vein (Fig. 4). The liver of rats treated with NSAIDs showed marked centrilobular necrosis in hepatocytes, with marked mononuclear cell infiltration (Figs. 5 and 6). The necrotic hepatocytes were characterized by cell enlergment and nuclear dissolution. In the vitamin C and E supplemented groups the degree of necrosis and infiltration was less extensive than that in liver from rats treated with NSAIDs (Figs. 7 and 8).

Liver is a versatile organ in the body concerned with regulation of internal chemical environment. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. Both vitamin C and E may play an important role in physiological reactions such as mixed function oxidation involving incorporation of oxygen into a biochemical substrate. In addition, these vitamins are considered the most important antioxidant in extra cellular fluids and its antioxidant function has been shown to efficiently scavenge superoxide, hydrogen peroxide, hydroxyl, peroxyl and singlet oxygen radicals. Vitamin C and E can efficiently scavenge free

TABLE-1
EFFECT OF VITAMIN C AND E ON DIFFERENT BIOCHEMICAL PARAMETERS OF NSAIDs INDUCED HEPATOTOXICITY IN RATS

Groups	ALT	AST	ALP	Bilirubin		Cholesterol	BUN
				Direct	Total		
Group I	17.63 ± 1.29	52.29 ± 1.26	36.60 ± 1.93	0.09 ± 0.02	0.84 ± 0.02	61.57 ± 1.30	19.40 ± 1.07
Group II	68.34 ± 1.34*	92.27 ± 0.89*	54.06 ± 2.34#	0.74 ± 0.03*	6.26 ± 0.28*	39.60 ± 1.20#	29.46 ± 0.72*
Group III	24.26 ± 2.14**	69.18 ± 1.98##	38.58 ± 1.24**	0.25 ± 0.08**	1.20 ± 0.26##	59.90 ± 1.22**	22.74 ± 1.33**
Group IV	72.29 ± 0.92*	98.43 ± 1.32*	57.14 ± 3.68#	0.69 ± 0.03*	5.94 ± 0.24*	41.26 ± 1.21#	32.57 ± 0.94*
Group V	28.19 ± 1.92	65.02 ± 2.04##	42.74 ± 2.49**	0.27 ± 0.09**	1.00 ± 0.09##	60.34 ± 1.19**	21.81 ± 1.94**

All values are mean ± SEM n = 6 rats in each group; *p < 0.001, #p < 0.05 as compared with Group-I **p < 0.001, ##p < 0.05 as compared with Group-II and group IV.

TABLE-2
EFFECT OF VITAMIN C AND E ON LIPID PEROXIDATION, ANTIOXIDANT ENZYMES AND GSH IN LIVER OF NSAIDs INDUCED HEPATOTOXICITY IN RATS

Groups	SOD	CAT	GSH	GPx	Lipid peroxidase
Group I	54.74 ± 2.94	76.29 ± 1.26	97.76 ± 2.76	34.58 ± 1.62	5.40 ± 2.52
Group II	39.81 ± 3.91*	51.74 ± 1.46#	50.76 ± 2.76*	21.74 ± 1.24*	8.71 ± 1.38
Group III	48.54 ± 2.54**	69.74 ± 2.81**	86.81 ± 2.79**	36.24 ± 1.92##	6.14 ± 2.51**
Group IV	32.29 ± 1.70*	46.81 ± 2.59#	61.27 ± 3.86*	19.89 ± 2.68*	9.02 ± 2.45#
Group V	50.86 ± 1.32**	71.84 ± 3.12**	94.46 ± 2.12**	30.66 ± 2.51##	6.06 ± 1.64**

All values are mean ± SEM n = 6 rats in each group; *p < 0.001, #p < 0.05 as compared with Group-I **p < 0.001, ##p < 0.05 as compared with Group-II and Group IV.

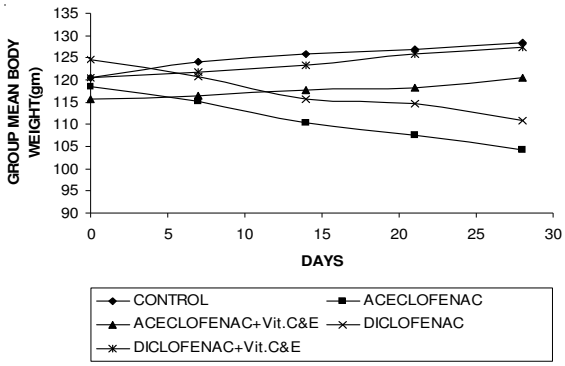

Fig. 1. Effect of vitamin C and E on mean body weight in NSAIDs induced hepatotoxiccity in rats

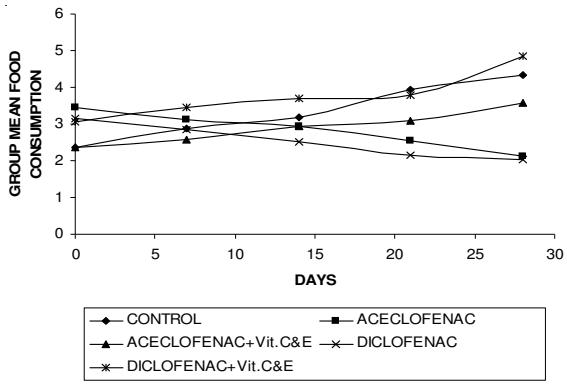

Fig. 2. Effect of vitamin C and E on food consumption in NSAIDs induced hepatotoxicity in rats

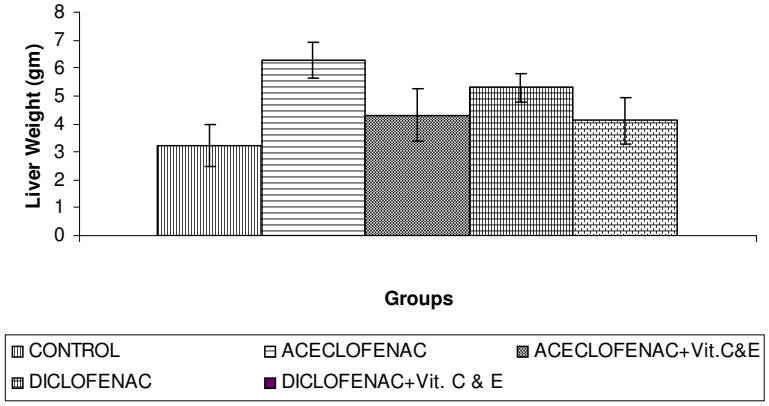

Fig. 3. Effect of vitamin C and E on liver weight in NSAIDs induced hepatotoxicity in rats

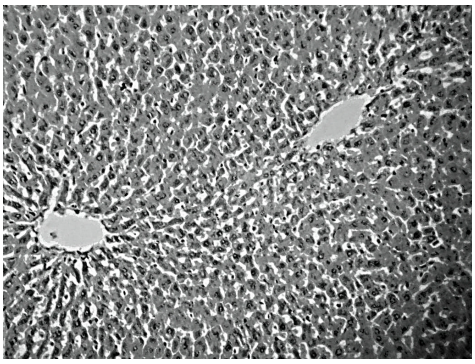

Fig. 4. Normal liver histology showing central vein, hepatocytes plates and portal tried

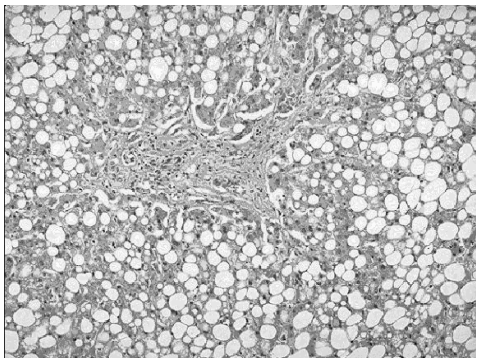

Fig. 5. Aceclofenac treated, features of fatty liver most of the hepatocytes are distended with large lipid vacuoles

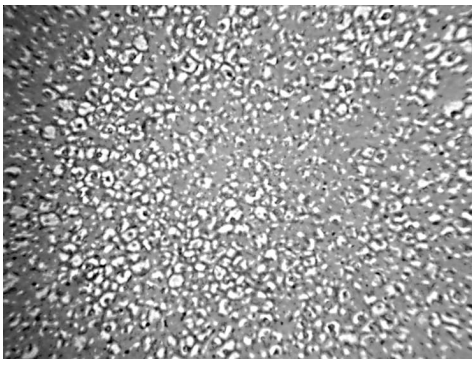

Fig. 6. Diclofenac treated, feature of fatty liver, absent of central vein

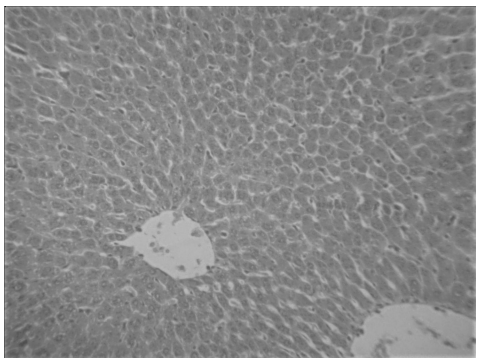

Fig. 7. Liver section treated with diclofenac + vitamin C&E showing normal histology, central vein and normal architecture

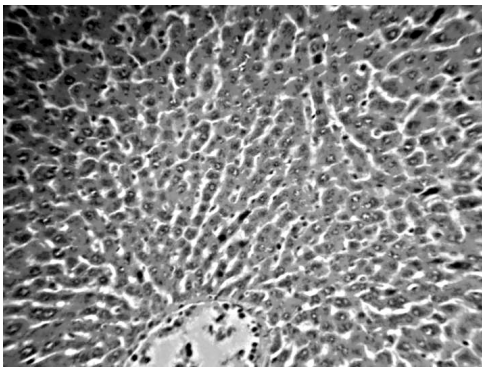

Fig. 8. Aceclofenac + vitamin C + E liver showing normal architecture

radicals before they can initiate lipid peroxidation and contribute to stability of cellular and basal membranes. The antioxidant vitamin C and E may suppress the hepatotoxic effects of aceclofenac and diclofenac by interference with intermediary metabolites. Antioxidants may prevent the harmful effects of free radicals and may suppress the formation of reactive intermediary metabolites of diclofenac sodium[17]. Formation of ROS, oxidative stress and hepatocellular injury has been implicated to NSAIDs induced liver injury. It has been documented that Kupffer cells are the major sources of ROS during chronic NSAIDs complication and these are primed and activated for enhanced formation of pro-inflammatory factors[19].

The activities of assay of aspertate transaminase (AST), assay of alanine transaminase (ALT), are the specific markers to assess hepatocellular damage leading to liver cell necrosis[20]. Slight to moderate increases in ALP (1-2 times normal) occurred in liver disorders[21].

As can be seen from the Table-1, aceclofenac and diclofenac produces serious liver injury indicated by significant elevation of ALT, AST, ALP, BUN, bilirubin and cholesterol ($p < 0.001$, $p < 0.05$) administration of vitamin C and E along with NSAIDs (Group III & IV) successfully controlled the rise in ALT, AST, ALP, BUN, bilirubin, cholesterol.

Oxidation of polyunsaturated fatty acids (lipid peroxidation) of membranes is a common process in living organism, since they are the target of oxygen-derived free radicals produced during mitochondrial electron transport[22]. Increased accumulation of lipid peroxidation products in cells can result in cellular dehydration, whole cell deformity and cell death[23]. In this study vitamin C and E co-administered rats showed significantly decreased levels of these lipids peroxidation markers as compared with experimental rats. The inhibition of lipid peroxidation by vitamin C and E, therefore, may be one of the mechanisms by which these vitamins exert its protection against NSAIDs mediated tissue injury.

Free radical scavenging enzymes, such as SOD and CAT, are the first line of defense against oxidative injury. SOD is ubiquitous cellular enzyme that dismutates superoxide radical to H_2O_2 and oxygen and is one of the chief cellular defense mechanisms. The H_2O_2 formed by SOD and other processes is scavenged by catalase that catalyzed the dismutation of H_2O_2 into water and molecular oxygen. Thus, the antioxidant enzyme catalase is responsible for detoxification of H_2O_2. The catalase enzyme may also be released into the extracelular environment in which it has the potential to function as a potent antioxidant and thereby regulated cell survival[24,25].

Histologically the liver from Group I animals revealed a normal architecture. Histopathology of aceclofenac and diclofenac treated livers indicated hepatotoxicity in terms of fatty infiltration and necrosis. Administration of vitamin C and E were able to control this necrotic changes comparable to treated groups. Thus, the biochemical observations correlated well with the histopathology results of the liver samples.

In conclusion, we determined that aceclofenac and diclofenac could increase the liver enzyme levels and affect some hepatospecific biochemical parameters. Increase in these parameters may occur due to peroxidation reactions and these reactions may inflict oxidative injury to cellular components. In the light of these results, combination of vitamin C and E may play a role in the prevention of hepatic cellular injury produced by non steroidal anti-inflammatory drugs.

It may be concluded that combination of vitamins C and E to be even more effective than C alone in helping the body repair cell damage due to NSAIDs induce liver damage.

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