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Full Length Research Paper

Protective effect of green tea on CCl_4 induced hepatoxicity in experimental rats

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Liver diseases are among the major health problems worldwide. Despite their increasing frequency, high morbidity and mortality, medical management is supposed to be insufficient; no therapy has successfully prevented the progression of hepatic diseases. Green tea is one of the commonly used beverages and considered as powerful antioxidant. This study was designed to investigate the protective effects of green tea administration on CCl_4 induced liver cirrhosis in experimental rats. A total of 24 male Wistar rats were selected and divided into 4 experimental groups (6 each) as: Group-1: normal healthy untreated rats; Group-2: CCl_4 (0.8 mg/kg) induced cirrhotic rats; Group-3: treated with 5% green tea orally; Group-4: treated with CCl_4 (0.8 mg/kg) intraperitoneally once a week for 8 weeks+5% oral administration of green tea. The preventive effects of green tea were measured by means of plasma alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and direct bilirubin, tissue malondialdehyde (MDA), tissue superoxide dismutase (SOD) and tissue catalase. Induction of cirrhosis by CCl_4 was indicated by high levels of plasma ALT, direct bilirubin, tissue MDA and low levels of tissue SOD. Results showed that administration of green tea reduced these changes significantly in cirrhotic rats by putting beneficial effects on antioxidant and liver enzymes as well as total and direct bilirubin. This study convinced the possible protective effect of green tea in relation to antioxidant and liver enzymes. It is also conclusive that the chronic and sub chronic administration of green tea extracts has counter effects on hepatotoxicity caused by CCl_4 administration.

Key words: Green tea, total and direct bilirubin, cirrhosis, alanine aminotransferase (ALT), alkaline phosphatase (ALP).

INTRODUCTION

Liver diseases are among the major health problems worldwide. Despite their increasing frequency, high morbidity and mortality, medical management is supposed to be insufficient, no therapy has successfully prevented the progression of hepatic diseases, even though newly developed drugs have been used to treat chronic liver disorders, these drugs have often side effects (Wolf et al., 2008). Liver cirrhosis is a result of late stage scarring in chronic liver disorder. It is progressive damage to the liver tissue starting with subendothelial or hepatic fibrosis and develops with nodule formation, which is actually called cirrhosis (Richard et al., 2008). This inflammation is produced by free radicals generated by viruses, toxins, unhealthy fats, alcohol and some drugs or antibodies that are attacking liver cells (Tsukamoto et al., 1997).

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Administration of single or repeated dose of CCl_4 is one of the common methods to investigate the possible mechanisms of hepatic injury in rats. This model has been implemented in various studies for the deposition of extracellular matrix in the cases of liver cirrhosis and fibrosis (Luckey and Petersen, 2001; Nakade et al., 2002). The biotransformation process of CCl_4 causes the formation of haloalkane free radicals which can damage the hepatocytes making liver an important target for CCl_4 (Weber et al., 2003; Ozardali et al., 2004). Herbal medicine derived from plant extracts is increasingly used to treat various medical problems. Plant extracts have been used by traditional medical practitioners for the treatment of liver disorders for centuries. It is being acknowledged that plants contain non-nutritional constituents with beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties (Amuta et al., 2010). A number of previous studies suggested that oxidative stress and DNA damage is responsible to initiate the tumor formation and the normal process of oxidation produces highly reactive free radicals (Kyung et al., 2007).

Green tea, one of the popular beverages of the world, contains polyphenolic antioxidants, which are thought to contribute to cancer prevention (Jin et al., 2008). Although, an association between these beverages and liver cancer has been speculated, epidemiologic evidence is insufficient and varies by beverage (WCRF, 2007). *Camellia sinensis* (Tea) exhibits a wide range of effects on human and animal health. It is anti-inflammatory (Dona et al., 2003) and has beneficial effects in collagen-induced arthritis, inflammatory bowel disease and carrageenan-induced paw edema (Das et al., 2002). An increased consumption of green tea may reduce the risk of liver disease (Jin et al., 2008). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. These free radicals readily react and damage biomolecules and DNA. Green tea polyphenol prevents oxygen free radical-induced hepatocyte lethality, prevent lipopolysaccharide-induced liver injury through inhibition of inducible nitric oxide synthase and tumor necrosis factor-α expression and inhibits carcinogen or toxin-induced liver oxidative DNA damage (Cai et al., 2002). Catechin isolated from green tea, has antioxidant properties and is thought to act as an antioxidant in biological systems. The protective effects of tea extracts or tea polyphenol against liver fibrosis and liver cirrhosis in rats have been reported. The polyphenols contained in the tea are antimutagenic and anticarcinogenic by inhibiting cancer cell proliferation and induction of apoptosis (Bun et al., 2006).

By keeping in mind the beneficial effects of green tea administration on liver damage, this study was conducted to evaluate the hepatoprotective effects of green tea against CCl_4 induced cirrhosis in experimental rats.

MATERIALS AND METHODS

Study animals

A total of 24 male albino Wistar rats weighing 200 to 250 g body weight (g.b.w), purchased from the animal house of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi were selected for the study. Rats were acclimatized according to the laboratory environment more than one week before the commencement of experiment. All the rats were caged with saw dust covered floor, in a quiet and temperature controlled room (23 ± 4°C). The rats were given free access to stan dard rats' diet and water. All the protocols regarding this study were approved by institutional ethical committee and conducted according to the ethical guidelines for the use of animals in laboratory experiments.

Study design

The age and sex-matched rats were randomly divided into 4 experimental groups consisting of 6 rats per group: Group-1 contained controls, fed on standard diet and water; Group-2 contained rats treated with CCl_4 (0.8 mg/kg b.w., i.p), the dose was given intraperitoneally at 11:45 a.m., once a week for 8 weeks; Group-3 contained rats that received 5% green tea extract prepared in distilled water orally on daily basis, the volume of green tea extract consumed by each rat was measured on 11:30 a.m. every morning, and the mean intake of green tea extract was 48.7 ± 12.56 ml on the day-1, which was increased to 120.3 ± 8.86 ml on day-45 of treatment; Group-4 contained rats that received CCl_4 (0.8 mg/kg b.w., i.p) weekly for 8 weeks as well as green tea extract (5%) orally on daily basis, the volume of green tea consumed by each rat was measured on 11:30 a.m. every morning, and the mean intake of green tea extract in these rats was 40.5 ± 12.56 ml on day-1, which was increased to 110.5 ± 15.45 ml on day-45 of treatment.

Collection of sample

After 8 weeks treatment, animals were decapitated and blood samples were collected from head wound in the lithium heparin coated tubes. A portion of blood was separated to collect the plasma. Liver were excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissues were then kept in freezer at -80°C until analysis.

Preparation of liver homogenate

A portion of liver was weighed, perfused with saline and homogenized in chilled potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 min at 4°C to get the post mitochondrial supernatant which was used to assay superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) activities.

Estimation of liver enzymes

Plasma alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and direct bilirubin were analyzed using commercially available reagent kits from Randox® Laboratories Ltd. UK (Reitman and Frankel, 1957).

Estimation of CAT activity

CAT activity was assayed by the method of Sinha et al. (1972). Briefly, the assay mixture consisted of 1.96 ml phosphate buffer (0.01 M, pH 7.0), 1.0 ml hydrogen peroxide (0.2 M) and 0.04 ml phenazine methosulfate (PMS) (10%) in a final volume of 3.0 ml. About 2 ml dichromate acetic acid reagent was added in 1 ml of reaction mixture, boiled for 10 min, and was cooled. Changes in absorbance were recorded at 570 nm.

Estimation of SOD

Levels of SOD in the cell free supernatant were measured by the method of Kono (1978). Briefly, 1.3 ml of solution A (0.1 mM ethylenediaminetetraacetic acid (EDTA) containing 50 mM Na_2CO_3, pH 10.5), 0.5 ml of solution B (90 mm nitro blue tetrazolium (NBT) dye), 0.1 ml of solution C (0.6% TritonX-100 in solution A) and 0.1 ml of solution D (20 mM hydroxylamine hydrochloride, pH 6.0) was mixed and the rate of NBT reduction was recorded for 1 min at 560 nm. 0.1 ml of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described earlier. One enzyme unit was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate by 50% in 1 min.

Assessment of tissue lipid peroxidation

Butylated hydroxytoluene (BHT) of 10 µl (0.5 M in acetonitrile) was added to prevent homogenate from oxidation and the homogenate was stored at -70℃ until analysis for MDA.

Estimation of MDA

The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) (Ohkawa et al., 1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of PMS. The mixture was brought up to 4.0 ml with distilled water and heated at 95℃ for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its activity was measured at 532 nm and was compared with those obtained from MDA standards. The concentration values were calculated from absorption measurements as standard absorption.

Estimation of total and direct bilirubin

The total and direct bilirubin was estimated by previously described method (Jendrassik and Grof, 1938).

Statistical analyses

The results are presented as mean ± standard error of the mean (SEM). Statistical significance and differences from control and test values were evaluated by Student's *t*-test. Statistical probability of $P<0.01$ and $P<0.05$ were considered as significant. All the analyses were done using Statistical Package of Social Sciences (SPSS) version 14.0 for Windows.

RESULTS

Results are presented in Tables 1 to 4. To measure activity of antioxidant enzymes, the activity of SOD was significantly low ($P<0.01$) in CCl_4 treated cirrhotic and green tea treated rats, whereas no difference was found in green tea treated cirrhotic rats as compared to the control rats. The activity of CAT was found to be significantly low ($P<0.05$) in CCl_4 treated cirrhotic rats. No change was observed in the activity of CAT in green tea treated as well as green tea treated cirrhotic rats as compared to the controls (Table 1). The activities of liver enzymes (ALT and ALP) were found to be disturbed in CCl_4 treated rats. The activity of both ALT and ALP was significantly high ($P<0.01$) in CCl_4 treated cirrhotic rats as compared to control rats. Higher activity ($P<0.01$) of ALT was observed in green tea treated cirrhotic rats as compared to the controls, whereas no significant difference was observed in the activity of ALP in green tea treated cirrhotic rats (Table 2). The level of total bilirubin was found to be significantly high ($P<0.01$) in CCl_4 treated cirrhotic rats, whereas it was low ($P<0.05$) in green tea treated cirrhotic rats as compared to control rats. The direct bilirubin level was also observed and found to be high ($P<0.01$) in CCl_4 treated cirrhotic and green tea treated cirrhotic rats as compared to control rats (Table 3).The tissue lipid peroxidation was measured by means of tissue MDA levels that was found to be significantly high ($P<0.01$) in CCl_4 treated cirrhotic and green tea treated cirrhotic rats as compared to controls. On the other hand, no significant change was observed in green tea treated rats (Table 4).

DISCUSSION

Green tea has been known for its antioxidant, antimutagenic, and anticarcinogenic properties. The other possible benefits include treatment of cardiovascular disease, diabetes, dermatological manifestations, obesity, and oral problems (Hara, 2001). Green tea catechins have been found to have a number of antioxidant activities, including scavenging of such reactive oxygen species as superoxide, hydroxyl and peroxyl redicals, inhibition of lipid oxidation and inhibition of oxidation of low-density lipoproteins. Green tea may be chemopreventive agent for hepatocarcinogenesis in the absence of chronic hepatocyte damage (Luper, 1999).

This study describes the low levels of SOD in cirrhotic group (Table 1), which indicates a disturbance in free radical levels and thereby an increase in cellular damage. The amount of free radicals generated and the antioxidants present in the cell are imbalanced. As cirrhosis is supposed to be the last stage of liver cancer, therefore new cells were not synthesized and SOD was not produced, indicated by the low levels of SOD in cirrhotic rats in this study. The activity of SOD in liver

Table 1. Effects of green tea on SOD and CAT activity in CCl_4 induced liver cirrhosis in experimental rats.

Parameter	Group-1 (Control)	Group-2 (CCl_4 treated rat)	Group-3 (Green tea treated rat)	Group-4 (CCl_4 + Green tea treated rat)
SOD (U/gm of tissue)	26.08 ± 2.9	9.37 ± 0.9*	18.57 ± 1.1*	21.4 ± 3.8
CAT (mmol/g of tissue)	3.97 ± 0.3	1.505 ± 0.3**	3.24 ± 0.5	2.739 ± 1.6

Table 2. Effects of green tea on liver enzymes in CCl_4 induced liver cirrhosis in experimental rats.

Parameter	Group-1 (Control)	Group-2 (CCl_4 treated rat)	Group-3 (Green tea treated rat)	Group-4 (CCl_4 + Green tea treated rat)
ALT (IU/L)	52.55 ± 3.4	896.49 ± 39.1*	69.91 ± 9.5**	487.73 ± 18.7*
ALP (IU/L)	484.16 ± 19.1	947.16 ± 27.1**	362.15 ± 21.5**	528.83 ± 69.6

Table 3. Effects of green tea on total and direct bilirubin in CCl_4 induced liver cirrhosis in experimental rats.

Parameter	Group-1 (Control)	Group-2 (CCl_4 treated rat)	Group-3 (Green tea treated rat)	Group-4 (CCl_4 + Green tea treated rat)
Total bilirubin (µmol/L)	13.45 ± 2.7	24.82 ± 0.9*	11.90 ± 1.3**	8.87 ± 0.3*
Direct bilirubin (µmol/L)	4.32 ± 1.1	16.77 ± 3.5*	3.45 ± 0.4**	6.58 ± 0.7*

Table 4. Effects of green tea on tissue lipid peroxidation in CCl_4 induced liver cirrhosis in experimental rats.

Parameter	Group-1 (Control)	Group-2 (CCl_4 treated rat)	Group-3 (Green tea treated rat)	Group-4 (CCl_4 + Green tea treated rat)
MDA (nmol/g of tissue)	1.09 ± 0.3	2.17 ± 0.6*	0.84 ± 0.2	1.83 ± 0.5*

Values are mean ± SEM. Significant difference between controls and test groups by Students' *t*-test. *p < 0.01, as compared to controls.

cancer cells was a negative feedback of the multiplication of cancer cells and loss of lipid peroxidation explains the malignancy of hepatocarcinoma, and enhanced lipid peroxidation in liver cancer cells may cause the necrosis (Alia et al., 2006; Bechman and Koppenol, 1996).

In the present study, the levels of SOD in green tea treated rats were significantly low (P<0.01) as compared to controls, while the combined effect of CCl_4 and green tea suggested higher levels of SOD non-significantly. Applications of antioxidants that elevates SOD expression or treatment with SOD mimetic suppresses tumorigenesis both *in vivo* and *in vitro* (Elchuri et al., 2005).

The low level of catalase activity was observed in cirrhotic rats (Table 1) that received only CCl_4 which has decreased the antioxidant status along with induction of cirrhosis. Low activity of antioxidant substances in patients with hepatocellular carcinoma is considered as an indicator to the distortion of oxidant-antioxidant balance and the decrease in antioxidant system efficiency (Reddy et al., 2011). This shows the participation of free radical in the processes of tumor pathogenesis. Green tea extracts produce H_2O_2 in a weak alkaline medium. This stabilizes the integrity of cell membrane keeping the membrane intact and enzymes enclosed in cirrhosis (Lenaz, 2001). The levels of catalase were non-significantly high in green tea treated rats. The green tea polyphenol possesses promising anticancer potential, while endogenous catalase may play a role in response to H_2O_2-induced cytotoxicity. The cytotoxic effects on tumor cells mainly result from sources other than H_2O_2. H_2O_2 accumulation can lead to reduction in cleavage of bonds between oxygen atoms leading to the production of hydroxyl radical, which is a very reactive and unstable oxidizing species that reacts instantaneously with any biological molecule. H_2O_2 can penetrate all biological membranes and can therefore cause damage in cellular locations far away from its

place of origin. That may be reason why the combined effects of green tea and CCl_4 suggested the higher levels of catalase activity in cirrhotic rats as compared to the controls.

This study also indicates an altered liver enzyme activity (ALT and ALP) (Table 2) and total and direct bilirubin levels (Table 3) which strongly indicates liver tissue injury. The increased serum levels of ALT and ALP have been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage. The ALT level was significantly increased in CCl_4 treated rats where the cells of liver have been inflamed and ALT leaked into blood stream, while ALP is significantly decreased in CCl_4 treated rats. ALP is synthesized in the bile canalicular cells and appears in the blood stream only whenever biliary duct is inflamed or blocked. It might be possible that CCl_4 produced only hepatic damage not biliary. Low ALP level is associated with magnesium deficiency as ALP activity is almost inhibited due to chelation of zinc and magnesium, the enzyme cofactors (Zaidi et al., 2005). Direct bilirubin concentrations was elevated in CCl_4 treated rats, indicating that it may be due to an increased production, decreased uptake by the liver, decreased conjugation, decreased secretion from the liver or blockage of bile ducts (Bun et al., 2006), decreasing amount of reducing equivalents, that is, NADPH reductase, reduced glutathione (GSH). GSH maintains the integrity of red blood cells (RBCs) membrane; its reduced level increases the hemolysis and increases the bilirubin level. Green tea increases the biliary flow and bile helps to eliminate the bile salts, fats, and toxins from the body. Herbal polyphenolic compounds in the cell can function as an antioxidant and prooxidant by scavenging reactive oxygen species via enzymatic and non-enzymatic reactions (Pyo et al., 2004).

The level of MDA in cirrhotic rats was found to be high as compared to the controls. Green tea treated group showed low levels of MDA (Table 4) as polyphenol rich green tea extracts inhibit lipid peroxidation in experimental rats as well as in human (Zaidi et al., 2005). During this study, the antioxidant system of cirrhotic rats was severely impaired, causing a high level of MDA in cancer tissues as compared to controls. The oxidative tissue damage in cirrhosis causes a significantly low level of catalase. During the process of inflammation oxidative stress occurs which leads to a significant decrease in antioxidant enzyme system. The main target of oxidative stress is the poly unsaturated fatty acids in cell membranes causing lipid peroxidation and excessive formation of MDA which may lead to damage of the cell structure and function (Kuper et al., 2000).

The results of this study are convincing that the administration of green tea has beneficial effects in CCl_4-incuded liver cirrhosis in experimental rats. The protective benefits of this herbal extract may be due in part to their potential antioxidant properties and ability to reduce oxidative stress. These should be supplemented with diet over a significant period of time to reduce the risk of hepatic damage caused by free redicals. In conclusion, these herbal products may be used for protective purpose which expose to hepatotoxic agents. However, more detailed studies are sought to refine the understanding of relationship between the hepatotoxicity and protective effects of herbal antioxidants.

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