Effects of green tea on ACE gene expression in rat liver in CCl₄-induced cirrhosis

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Aim: This study was designed to investigate the beneficial effects of green tea administration on angiotensin converting enzyme (ACE) gene expression in CCl₄-induced liver cirrhosis in rats.

Materials and methods: A total of 24 male Wistar rats were divided into 3 groups as: Group 1, normal untreated rats; Group 2, CCl₄-induced cirrhotic rats; Group 3: CCl₄-induced cirrhotic + green tea treated rats. The beneficial effects of green tea were measured by alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and direct bilirubin level, tissue malondialdehyde (MDA), super oxide dismutase (SOD), and catalase (CAT). The genomic DNA was isolated from excised tissue to determine the ACE genotypes using specific primers. The ACE gene expression in liver tissue was assessed using the quantitative RT-PCR method.

Results: Liver cirrhosis was indicated by high plasma ALT, direct bilirubin level, tissue MDA, and low SOD. The antioxidant enzymes SOD and CAT were low (P < 0.01) in cirrhotic and green tea-treated rats. High activity (P < 0.01) of ALT was observed in green tea-treated cirrhotic rats. The total and direct bilirubin levels were high (P < 0.01) in CCl₄-treated cirrhotic rats while they were low (P < 0.05) in green tea-treated cirrhotic rats. The tissue MDA was high (P < 0.01) in CCl₄ and green tea-treated cirrhotic rats. ACE gene expression after 8 weeks of CCl₄ treatment in cirrhotic rats was significantly high (P < 0.05), and was reversed in green tea-treated cirrhotic rats in comparison to controls.

Conclusion: The administration of green tea tries to correct the deteriorative biochemical and genetic changes during CCl₄-induced liver cirrhosis in rats. The long-term consumption of green tea has beneficial effects on abnormally increased ACE gene activity during liver cirrhosis caused by CCl₄ administration in rats.

Key words: Green tea, liver cirrhosis, ALT, ALP, total and direct bilirubin, ACE gene

1. Introduction
Liver problems are a major health issue globally. The management of these problems seems inadequate, looking at their high frequency and their morbidity and mortality rates. It is now well documented that the management and treatment strategies being used for hepatic disorders produce severe adverse effects (1). Liver scars and progressive damage to the liver tissue start with subendothelial or hepatic fibrosis, and develop with nodule formation. This condition may be caused by unhealthy fats, alcohol, toxins, and viruses, as well as some therapeutic agents that can affect liver cells and produce cirrhosis (2).

Angiotensin converting enzyme (ACE) is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells (3). The renin angiotensin system (RAS) is a cascade of the circulatory system basically involved in the regulation of blood pressure and water–electrolytes balance (4,5). ACE is the key enzyme in this system, which converts angiotensin I to the potent vasoconstrictor angiotensin II (6). The RAS is known to play a role in the pathophysiology of various diseases, including fibrosis in the lung, kidney, and heart, during chronic inflammation through the regulation of cell growth, inflammation, oxidative stress, angiogenesis, fibrosis, and cirrhosis (7). The ACE gene expression and its role as a potential disease marker have been investigated in several diseases (8). However, the abnormalities in expression of the ACE gene during liver injury and cirrhosis, with its contribution to the development of disease, have not yet been defined.

The development and progression of liver cirrhosis in experimental animals are widely studied by CCl₄ administration. The majority of cases, including liver
cirrhosis and fibrosis, have implemented these models for
deposition of extracellular matrix (9). The haloalkane free
radicals, which are formed during the biotransformation
process of CCl₄, can damage the hepatocytes, making the liver an important target for CCl₄ (10).

The use of plant extracts, in the form of herbal medicine,
is being exploited to tackle various medical issues. A
large number of beneficial health effects are reported
to be produced by these medicinal plants, including
antibacterial, antifungal, antitumor, antihypertensive,
antidiabetic, and antiinflammatory effects (11–13).

Camellia sinensis, commonly called green tea, exhibits
a wide range of beneficial effects on human and animal
health. It contains antioxidants like polyphenols, which
contribute to the prevention of cancers (14). It has beneficial
effects in collagen-induced arthritis, inflammatory bowel
disease, and paw edema (15). The long-term consumption
of green tea has been reported to greatly reduce the risk
of liver injury (16). The cellular damage and abnormal
levels of antioxidants are caused by oxidative stress and
cellular damage leading to DNA damage. The polyphenols
contained in the tea are antimutagenic and anticarcinogenic
due to their inhibition of cancer cell proliferation and
induction of apoptosis (17). By considering the above-
mentioned facts, this study was conducted to evaluate the
beneficial effects of green tea consumption on ACE gene
expression in CCl₄-induced liver cirrhosis in rats.

2. Materials and methods

2.1. Study design and animals

A total of 24 male albino Wistar rats (200–250 g body
weight), purchased from the animal house of the
International Center for Chemical and Biological Sciences,
University of Karachi, were selected for the study. The
rats were acclimatized to the laboratory environment for
1 week before the commencement of the experiment.
All the rats were caged with a sawdust-covered floor in a
quiet and temperature-controlled room (23 ± 4 °C). Rats
were given free access to a standard rat diet and water.
All the protocols regarding this study were approved
by the institutional ethical committee and conducted
according to the ethical guidelines for the use of animals
in laboratory experiments. The age- and sex-matched
rats were divided into 3 experimental groups (8 rats per
group): Group 1, the control, was fed on the standard diet
and water. Group 2 was treated with CCl₄ (0.8 mg/kg body
weight (b.w.), intraperitoneally (i.p.)). The dose was given
intraperitoneally at 1145 hours, once a week for 8 weeks.
Group 3 received CCl₄ (0.8 mg/kg b.w., i.p) weekly for 8
weeks as well as green tea extract (5 %) orally on a daily
basis. The volume of green tea consumed by each rat was
measured at 1130 hours every morning. The mean intake
of green tea extract in these rats was 45.5 ± 12.56 mL on
day 1, which was increased to 115.5 ± 15.45 mL on day 45.

2.2. Collection of samples

After 8 weeks of treatment, blood samples were collected by
decapitating the animals. After the trimming of connective
tissues, liver tissues were rinsed with saline to eliminate
blood contamination, dried, weighed, and then kept at −80
°C until analyzed.

2.3. Preparation of liver homogenate

A small piece of liver was weighed, perfused in saline, and
homogenized in ice-cold potassium chloride (1.17 %) with
the help of a homogenizer. The resultant was centrifuged
at 800 × g at 4 °C for 5 min in order to separate the nuclear
debris. The supernatant was centrifuged at 10,500 × g at 4
°C for 20 min to get the postmitochondrial supernatant.
This was used to estimate catalase (CAT), super oxide
dismutase (SOD), and malondialdehyde (MDA) activities.

2.4. Estimation of liver enzymes

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

2.5. Estimation of CAT activity

The previously described method of Sinha et al. (18) was
used to measure the CAT activity.

2.6. Estimation of SOD

The method of Kono (19) was used to estimate the activity
of SOD in the cell-free supernatant.

2.7. Assessment of tissue lipid peroxidation

A total of 10 µL of butylated hydroxytoluene (0.5 M
in acetonitrile) was added to prevent oxidation of the
homogenate, and the homogenate was stored at −70 °C
until analysis for MDA.

2.8. Estimation of MDA

The tissue lipid peroxidation was measured by means of
the MDA content. The method of Ohkawa et al. (20) was
used in the form of thiobarbituric acid reacting substances.

2.9. Estimation of total and direct bilirubin

The total and direct bilirubin level was estimated by a
previously described method (21).

2.10. Genotyping

Genomic DNA was isolated from excised tissue as described
previously (22). To determine the ACE genotypes, primers
were used, as described by Hilbert (23), to amplify the
microsatellite located at the 50 end of the intron between
exons 13 and 14.

2.11. ACE gene expression in liver tissue

The expression of ACE gene in liver tissue was assessed
using the quantitative reverse-transcription polymerase
chain reaction (RT-PCR) method. RNA was isolated using
Total RNA Prep Plus (A and A Biotechnology, Gdansk,
Poland). In brief, an amplification reaction was performed
in 12.5 µL of total volume, containing a pair of specific
primers: 5’ CAGCTTCATCATCCAGTTCC 3’ and 5’ CTAGGAAGAGCAGCACCAC 3’. The PCR program consisted of 30 cycles at an annealing temperature of 52–64 °C. Restriction fragments were subsequently analyzed in 2% agarose gel stained with the help of ethidium bromide (24) (Figure 1).

2.12. Statistical analyses
The results are shown as mean ± standard error of them mean (SEM). Statistical significance and differences between control and test groups were examined by Student’s t-test. Statistical probabilities of P < 0.01 and P < 0.05 were taken to be significant. All analyses were done using SPSS 17.0 for Windows.

3. Results
The activity of the antioxidant enzyme SOD was significantly lower (P < 0.01) in CCl₄-treated cirrhotic and green tea-treated rats, whereas no difference was found in green tea-treated cirrhotic rats when compared to controls. CAT activity was significantly lower (P < 0.05) in CCl₄-treated cirrhotic rats. No change was observed in the CAT level in green tea-treated cirrhotic rats as compared to the controls (Table 1).

A high ALT activity (P < 0.01) was observed in green tea-treated cirrhotic rats, whereas no significant difference was observed in the activity of ALP in green tea-treated cirrhotic rats compared to the controls (Table 2).

Total bilirubin was significantly higher (P < 0.01) in CCl₄-treated cirrhotic rats and lower (P < 0.05) in green tea-treated cirrhotic rats when compared to the controls. The direct bilirubin level was also high (P < 0.01) in CCl₄-treated cirrhotic and green tea-treated cirrhotic rats as compared to controls (Table 3).

The tissue lipid peroxidation, as measured by tissue MDA, was found to be significantly higher (P < 0.01) in CCl₄-treated cirrhotic rats and lower (P < 0.05) in green tea-treated cirrhotic rats compared to the controls (Table 4).

Figure 2 shows the expression of the ACE gene in the experimental groups. The expression of the ACE gene after 8 weeks of CCl₄ treatment in cirrhotic rats was significantly increased (P < 0.05) in comparison with tissue samples from the control group. This gene expression was found to be reversed in the green tea-treated cirrhotic rats (Figure 2).

Table 1. Effects of green tea on SOD and CAT activity in liver damage in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Controls)</th>
<th>Group 2 (CCl₄-treated rats)</th>
<th>Group 3 (CCl₄ + green tea-treated rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/g)</td>
<td>26.08 ± 2.84</td>
<td>9.37 ± 0.88*</td>
<td>21.4 ± 3.77</td>
</tr>
<tr>
<td>CAT (mmol/g)</td>
<td>3.97 ± 0.34</td>
<td>1.505 ± 0.24**</td>
<td>2.739 ± 1.53</td>
</tr>
</tbody>
</table>

In all tables, values are mean ± SEM. Significant difference between controls and test groups was determined by Student's t-test; *: P < 0.01, **: P < 0.05 as compared to controls.

Table 2. Effects of green tea on liver enzymes in liver damage in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Controls)</th>
<th>Group 2 (CCl₄-treated rats)</th>
<th>Group 3 (CCl₄ + green tea-treated rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>52.55 ± 3.39</td>
<td>896.49 ± 39.08*</td>
<td>487.73 ± 18.64*</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>484.16 ± 19.06</td>
<td>947.16 ± 27.08**</td>
<td>528.83 ± 69.52</td>
</tr>
</tbody>
</table>
4. Discussion

Green tea has been reported to produce beneficial effects for the treatment of cardiovascular disease, diabetes, dermatological manifestations, obesity, and oral problems (25). The antioxidant activities, including scavenging of such reactive oxygen species (ROS) as superoxide, hydroxyl, and peroxyl radicals; inhibition of lipid oxidation; and inhibition of low-density lipoprotein oxidation have been reported to be the properties of the catechins found in green tea. Chemoprotection has been considered as another important feature of green tea that prevents carcinogenesis in liver damage (26).

This study describes the low levels of SOD in a group of rats with cirrhosis of the liver (Table 1), indicating an imbalance in free radicals leading to cellular damage. The administration of CCl₄ caused liver injury; as a result new cells were not synthesized and SOD was not properly formed, which caused the low levels of SOD in cirrhotic rats. The negative feedback caused by the multiplication of cancer cells is an activity of SOD in cirrhotic cells. The loss of lipid peroxidation describes the malignancy of hepatocarcinoma and enhanced lipid peroxidation in liver cells, which may lead to necrosis (27).

The levels of SOD in green tea-treated rats were significantly lower (P < 0.01) when compared to the controls, while the combined effect of CCl₄ and green tea resulted in a nonsignificantly higher level of SOD. Antioxidants are usually found to elevate SOD expression while SOD mimetic suppresses tumorigenesis both in vivo and in vitro (28).

The cirrhotic rats that received CCl₄ showed a low level of catalase activity (Table 1) that induces liver injury by lowering the antioxidant status. The undermining of antioxidant substances in patients suffering from hepatocellular carcinoma is reported as an indicator of a distortion of the oxidant–antioxidant balance and the decrease in antioxidant system efficiency (29); thus, the participation of free radicals is established in the pathophysiology of carcinoma. H₂O₂ is produced by green tea extracts in a weak alkaline medium. Intact membranes and embedded enzymes are responsible for integrating the cell membrane in cirrhosis. The polyphenol found in green tea also has cancer preventing potential, whereas endogenous CAT plays a pivotal role in H₂O₂-produced cytotoxicity. The accumulation of H₂O₂ prevents the cleavage of bonds between oxygen that may lead to excessive hydroxylation and production of highly reactive and unstable oxidizing species that can readily react with any biomolecule. Almost all biological membranes are penetrated by H₂O₂, and can be damaged in various cellular locations far from its original point. That may be the reason why the combined effects of green tea and CCl₄ caused higher levels of CAT activity in hepatotoxic rats, as compared to the controls.

### Table 3. Effects of green tea on total and direct bilirubin in liver damage in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Controls)</th>
<th>Group 2 (CCl₄-treated rats)</th>
<th>Group 3 (CCl₄ + green tea-treated rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bilirubin (µmol/L)</td>
<td>13.45 ± 2.61</td>
<td>24.82 ± 0.83*</td>
<td>8.87 ± 0.28*</td>
</tr>
<tr>
<td>Direct bilirubin (µmol/L)</td>
<td>4.32 ± 1.09</td>
<td>16.77 ± 3.34*</td>
<td>6.58 ± 0.67*</td>
</tr>
</tbody>
</table>

### Table 4. Effects of green tea on tissue lipid peroxidation in liver damage in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group-1 (Controls)</th>
<th>Group-2 (CCl₄-treated rats)</th>
<th>Group-3 (CCl₄ + green tea treated rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>1.09 ± 0.26</td>
<td>2.17 ± 0.61*</td>
<td>1.83 ± 0.48*</td>
</tr>
</tbody>
</table>
Altered liver enzyme activity (ALT and ALP) (Table 2) and total and direct bilirubin (Table 3) were also observed in this study, which strongly suggested liver injury. The damaged structural integrity of the liver is mainly caused by the increased serum ALT and ALP located in cytosol that, after cellular damage, are released into main circulation. The ALT was significantly increased in CCl4-treated rats where the cells of liver were inflamed and ALT leaked into the blood stream, while ALP was significantly decreased in CCl4-treated rats. ALP was synthesized in the bile canalicular cells and appeared in the blood stream only whenever the biliary duct was inflamed or blocked. It might be possible that CCl4 produced only hepatic damage and not biliary damage. The magnesium deficiency is transcribed into a low ALP level, which is inhibited due to chelation of zinc and magnesium, enzyme cofactors (30). Direct bilirubin was elevated in CCl4-treated rats, which indicated elevated production and lower liver uptake, and lower conjugation, as well as secretion from the liver or biliary tract obstructions (17), decreasing amounts and lower conjugation, as well as secretion from the liver or biliary tract obstructions (17), decreasing amounts of reducing equivalents such as NADPH reductase, and reduced glutathione (GSH). GSH maintains the integrity of red blood cell membranes; its reduced level increases the presence of free radicals. It can be concluded that these kinds of herbal extracts may be used for protection upon possible exposure to hepatotoxicities. In the future, more focused studies will be of help to improve the knowledge regarding green tea extracts and their beneficial effects on liver damages and injuries.

References


