Chemoprevention by Lipid-soluble Tea Polyphenols in Diethylnitrosamine/Phenobarbital-induced Hepatic Pre-cancerous Lesions

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Abstract. *Background: Green tea polyphenols (GTPs) have been proposed as promising candidates for chemo-prevention. However, GTPs levels are maintained relatively low in the blood and are chemically-unstable. Lipid-soluble tea polyphenols (LTPs) are products of modified GTPs with ester linkage with fatty acids. LTPs are lipophilic and expected to provide improved absorption and utilization in the body compared with water-soluble polyphenols. The current study was designed to investigate the chemo-preventive property and the possible mechanisms of LTP action against diethylnitrosamine (DEN)-induced liver cancer in rats. Materials and Methods: Oral administration of LTPs at doses of 0, 40, and 400 mg/kg/day was initiated 2 weeks prior to DEN injection and was continued for 30 weeks. At that time point samples were collected and liver histopathological analyses were performed. Results: LTPs decreased the area and number of placental glutathione S-transferase-positive foci in liver samples of DEN-treated rats. Furthermore, LTPs counteracted DEN-induced fibrosis formation in liver. Immunohistochemical staining of rat liver showed that LTPs inhibited DEN-mediated elevations in numbers of cells positive for PCNA and 8-OHdG. Conclusion: For the first time, the present study demonstrated, that LTPs exert a chemo-preventive effect against precancerous lesions through inhibition of cellular proliferation and DNA damage in a rat liver model.*

Hepatocellular carcinoma (HCC) is the fifth most common cancer world-wide and the second leading cause of cancer mortality in men (1). Chronic infection with hepatitis B and C are major risk factors for HCC worldwide (2-4). Other factors that contribute to HCC formation include: exposure to environmental carcinogens such as aflatoxins, alcohol abuse, and genetic factors (5-7). Diethylnitrosamine (DEN) is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, polluted water, cosmetics, cured meat products, and pharmaceutical agents (8). DEN-induced HCC is an accepted and widely-used experimental model of hepatocarcinogenesis in humans (9).

At present, surgical resection is the treatment of choice for HCC in non-cirrhotic patients (10), but the recurrence rates can be as high as 50% within several years of surgery (11). There is no effective systemic chemotherapy currently available for the treatment of HCC, therefore, chemo-prevention should be considered for HCC prevention.

Phytochemicals have received much attention in the management of cancer (12). A large number of naturally-occurring agents have displayed chemo-preventive potential in a variety of animal models and human disease (13-14).

Green tea polyphenols (GTPs) are a group of naturally-derived plant compounds from tea leaves that may possess biologically-useful properties. Unfortunately, the digestive

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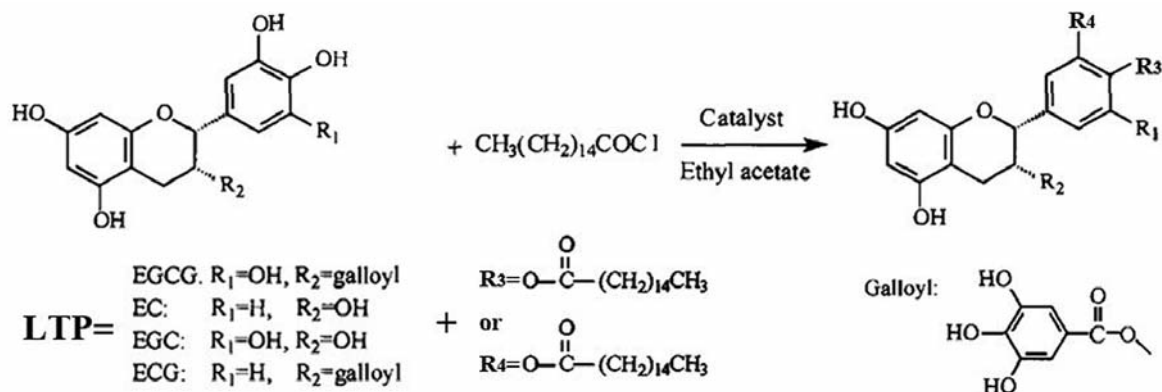*Key Words: GTPs, LTPs, precancerous lesions, proliferation, DNA damage.*

Figure 1. *Structure of Lipid-soluble tea polyphenols.*

system does not easily absorb GTPs. GTPs blood plasma concentration is usually less than 10 µM due to restricted absorption and high rates of catabolism (15-16). GTPs are also inherently unstable and easily oxidized. This property is part of what makes GTPs effective anti-oxidants.

The chemical constitution of GTPs were modified by esterification in an effort to solve the difficulties with absorption and chemical instability (17). This method produces lipid-soluble tea polyphenols (LTPs) (Figure 1), which can be dissolved in hydrophobic solvents. GTPs and their biologically-active compounds (including EGCG, ECG, EGC and EC) (18) have been shown both *in vitro* and *in vivo* to possess anti-oxidant, anticancer, and anti-inflammatory properties (19-28). This type of naturally-occurring compound could be potentially used as anti-oxidant food additive in various products, including dietary oils (17). The chemo-preventive effects of LTPs have not, however, been investigated against carcinogen-initiated hepatic neoplasia *in vivo*.

In order to elucidate the chemo-preventive action of LTPs, the current study used the well-described model of HCC to study the mechanism of the anticancer effects. This is the first reported attempt to demonstrate that LTPs significantly reduce the promotion of diethylnitrosamine (DEN)-induced hepatic pre-neoplastic lesions in rats.

Materials and Methods

Material and preparation. LTPs suspensions was purchased from Puremedie Biotechnology Co., Ltd. (Ningbo, Zhejiang, China) (11.5%, Production batches: 20091025-1), using corn oil as solvent. 67.2 ml LTPs were diluted to prepare the 80 mg/ml working mixture with 32.8 ml corn oil, and then diluted to 8 mg/ml working solution. The liquid was then administered to animals at doses of 0, 40, and 400 mg/kg of body weight in a volume of 0.5 ml/kg body weight.

Chemicals, kits and antibodies. N-Nitrosodiethylamine (DEN) was purchased from Sigma-Aldrich (St. Louis, MO, USA); γ-glutamyl transferase (GGT) was purchased from Nanking j-Jiancheng Biological Product Co.(Nanking, China); rabbit anti-GST-P primary antibody and anti-8-hydroxy-2'-deoxuguanosine (8-OHdG) antibody were purchased from Abcam (Cambridge, MA, USA); anti-proliferating cell nuclear antigen (PCNA) antibody from BioWorld Technology, Inc. (Louis Park, MN, USA); 3-diamino-benzidene (DAB) and immunohistochemical kits were purchased from Beijing Golden Bridge Biotechnology Company Ltd. (Beijing, china). Masson staining kit was purchased from Loogene Biotechnology Company Ltd. (Beijing, China).

Animals. All experiments involving animals were approved by the Laboratory Animal Use Committee of the Zhejiang University prior to experiments. Male Sprague- Dawley rats (147-157 g) were procured form the Zhejiang Academy of Medical Science (Hangzhou, Zhejiang, China). The animals were housed in solid-bottom polycarbonate cages (five animals/cage) under standard laboratory conditions (temperature 24±0.5˚C, relative humidity 55±5%, and a 12-h dark/light cycle). Animals were acclimatized to these conditions for two weeks prior to experiments.

Experimental design. Rats were divided to four groups, containing 25 animals each. Groups were subjected to the following treatments: Group 1 (Normal Control), animals were fed *ad libitum* throughout the experimental period and injected with a single dose of saline (0.9%). Group 2 (Solvent Control), DEN/PB-treated animals, were given LTPs 0 mg/kg by gavage 5-times weekly throughout the experimental period. Group 3 (LTPs 40 mg/kg), DEN/PB-treated animals, were given LTPs 40 mg/kg by gavage 5-times weekly throughout the experimental period. Group 4 (LTPs 400 mg/kg), DEN/PB-treated animals, were given LTPs 400 mg/kg by gavage 5-times weekly throughout the experimental period. HCC was induced in groups 2-4 by a single intraperitoneal injection of DEN, at 150 mg/kg of body weight after two weeks. After DEN administration, the promoter phenobarbital (PB) was incorporated into the drinking water of groups 2-4 at a concentration of 0.05%, which was consumed *ad libitum* by rats for up to 28 weeks (Figure 2).

Morphology, morphometry and histology. Rats were sacrificed 30 weeks post-treatment, livers were promptly excised, weighed and
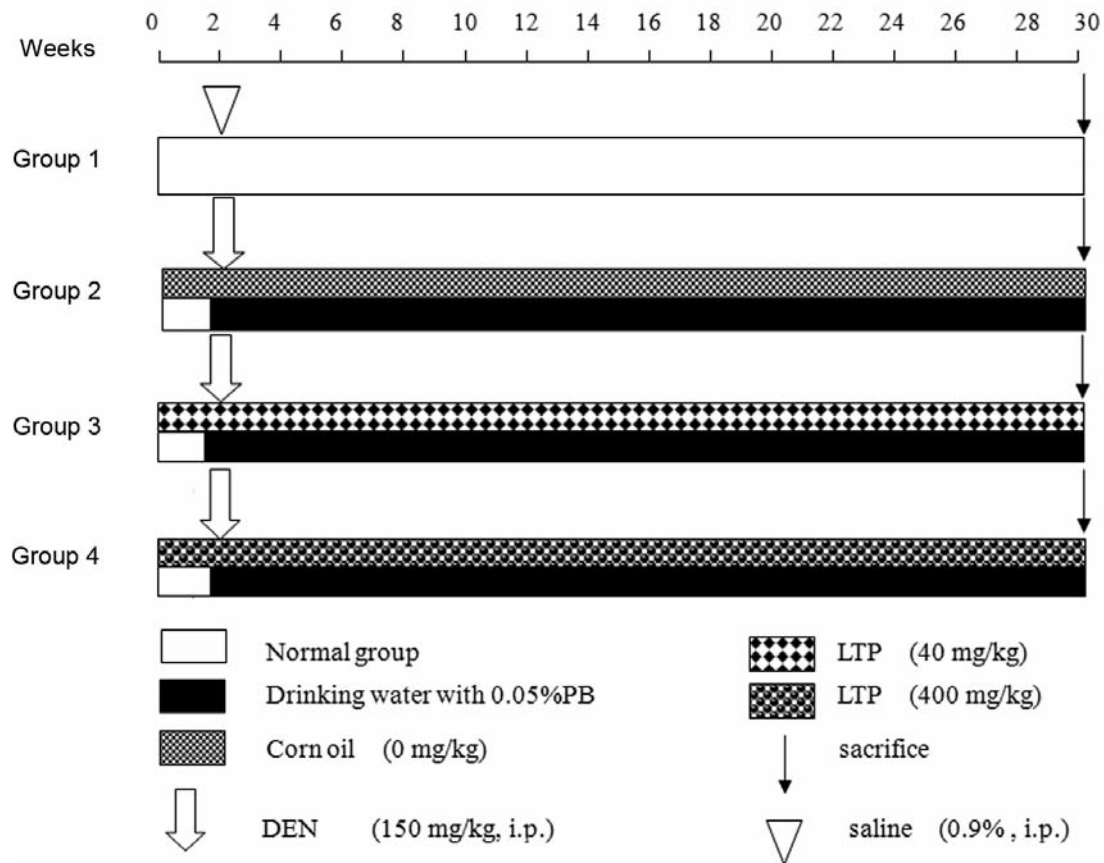
Figure 2. *Schematic representation of experimental procedure followed.*

macroscopically examined on their surface as well as in 3 mm cross-sections, for gross visible persistent nodules (PNs). Representative sections from right, left and caudate lobes of each liver were obtained, then fixed in 10% of neutral formaldehyde, paraffin embedded, sliced, and stained with H&E. Sections were then examined under light microscopy. Masson trichrome staining was performed to assess changes in collagen deposition and fibrosis. Scoring was established according to the following criteria (Table I) (29).

Immunohistochemistry (IHC). 5-μm sections of paraffin-embedded liver tissue were analyzed for expression of PCNA and 8-OHdG by immunohistochemistry. Briefly, sections were de-paraffinized in xylene and de-hydrated through graded ethanol. After washing with PBS three times, the sections were incubated with 3% hydrogen peroxide for 10 min at room temperature for inhibiting endogenous peroxidase activity. After rinsing in PBS three times, the sections were washed with citrate buffer solution (pH 7.2-7.6) for 15 min at 98°C. The sections were treated with 5% normal goat serum for 40 min to reduce non-specific binding. The sections were then incubated overnight with antibodies against 8-OHdG, PCNA, and GST-P. In the second day, sections were treated using an immunohistochemistry kit. Incubation with the appropriate secondary antibody was followed by direct diaminobenzidine staining and light counterstaining with hematoxylin.

The numbers and area of GST-P foci larger than 200 μm in diameter in the liver sections at the early stages of tumor promotion were measured as reported previously (30). The GST-P foci were counted in 5 randomly-selected fields under ×100 magnification. Then the number and areas of foci/cm2 were calculated. The brownish yellow nuclei particles represented the positive signal for PCNA, which were counted in 6 randomly selected fields under ×400 magnification. PCNA labeling index (LI) was expressed as the number of PCNA-positive hepatocytes×100/total number of hepatocytes analyzed. The brownish-yellow particles represented positive 8-OHdG, which were detected in 6 randomly selected fields under ×400 magnification. The relative expression was calculated using the Image-plus software (Media Cybernetics Inc., MD, USA).

Electron microscopy. The liver tissue was fixed in 2.5% glutaraldehyde buffered for 2 h, then stored at 4°C. It was fixed in 1% cold osmium tetraoxide for 1 h and washed using 0.1M PBS at pH 7.2 for 15 min. Ultrathin sections were obtained from specimens embedded in Lowicryl K4M resin after dehydration through a series of graded ethanol, substitution and polymerization at graded temperature series. Ultrathin sections were obtained using an Ultracut microtome (Leica, Vienna, Austria). Sections were mounted on 400-mesh collodion-carbon-coated nickel grids and examined with a Joel Electron Microscope (JAPAN) operating at 80 kV.

Table I. *Masson dye staging system (according to reference 29).*

Changes	Score
No fibrosis	0
Fibrous expansion of some portal area, with or without short fibrous septa	1
Fibrous expansion of most portal area, with or without short fibrous septa	2
Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3
Fibrous expansion of portal areas with marked bridging (P-P as well as portal to central (P-C))	4
Marked bridgeing (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5
Cirrhosis, probable or definite	6

Statistical analysis. Data was analyzed using SPSS 18.0 and presented as mean±S.E. Multi-group comparisons were evaluated using one-way analysis of variance (ANOVA) followed by Least-significant difference (LSD) in *post-hoc* test for the experiment groups. Statistical probability of $p<0.05$ was considered significant. Masson trichrome staining data were analyzed by the Kruskal-Wallis test.

Results

Body and liver weight. The mean body weight gain of different groups is presented in Table III. There was a decrease in the final body weight of LTPs 40- and 400-mg/kg groups compared to the 0-mg/kg group. The average liver weight of LTPs 0-mg/kg group was significantly increased compared to that of the normal control group ($p<0.05$). A similar correlation was found for the liver organ coefficients between the two groups. However, the liver weight and relative liver weight in the 400-mg/kg group was found to be significantly decreased compared to that of 0-mg/kg group ($p<0.05$). There was no statistical difference between the 40-mg/kg group and the 0-mg/kg group for liver weights and relative liver weights (Table III).

Effect of LTPs on hepatic histology. Histopathological analysis of liver sections from various experimental groups of animals is depicted in Figure 3. The livers of normal control animals (Group 1) showed normal hepatocellular architecture mainly consisting of normal cytoplasm and small uniform nuclei radially arranged in a radial pattern around the central vein of the hepatic lobules (Figure 3A). Animals subjected to DEN/PB and solvent (Group 2) showed a loss of abnormal architecture with irregularly-shaped hepatocytes and increased nucleoplasm ratios. Moreover, extensive steatosis cells with vacuoles in the cytoplasm were clearly distinguishable from the surrounding normal parenchyma (Figure 3B). Rats treated with LTPs at a dose of 40 mg/kg had only marginally-improved hepatocellular architecture compared to group 2 (Figure 3C). In group 4, the group receiving LTPs at 400 mg/kg, a moderate improvement in hepatocellular structure was evidenced.

Steatosis decreased significantly compared to group 2. The hepatocellular morphology, with regular arrangement and size of nuclei, was essentially the same as the one observed in normal cells.

Ultrastructural liver analysis. In electron microscopy preparations, the cell surface of the hepatic cells from the control group was smooth, with a large, spherical nucleus and nucleoli showing fibril granular network structure. The cytoplasm presented a granular appearance. There was a profuse amount of rough endoplasmic reticulum, especially around the nuclear envelope and between the rounded mitochondria. The hepatic sinusoids were thin-walled with discontinuous layer of endothelial and Kupffer cells. The endothelial cells were extremely thin with an electron-lucent cytoplasm (Figure 4A). In animals treated with LTPs 0 mg/kg, the nuclei were found to contain a large amount of scattered areas of heterochromatin. The cytoplasm of the hepatic cells contained a fairly large number of vacuole, fracturing of the rough and smooth endoplasmic reticulum, and many damaged mitochondria (Figure 4B). However, in the LTPs 400-mg/kg-treated rats, most cells had characteristic large-rounded nuclei and large nucleoli and contained relatively complete organelles, especially the rough endoplasmic reticulum and mitochondria. The cytoplasmic vacuoles were sparse and fat droplets were not observed in group 4 (Figure 4D).

Liver fibrosis. On Masson trichrome assay, the normal control group did not demonstrate histological evidence of steatosis, inflammation, or fibrosis (Figure 5A); whereas DEN/PB and solvent (Group 2) resulted in liver fibrosis (Table II). The 400-mg/kg group had mild fibrosis, with fibrosis scoring found to be statistically significant compare to Group 2 (Table III). LTP inhibited DEN-induced foci of altered hepatocyte formation and GST-p expression.

Glutathione S-transferase placental (GST-P) is specifically expressed during rat hepatocarcinogenesis, and has been used as a reliable tumor marker to monitor experimental hepatocarcinogenesis in rats (31-32). We, therefore, examined the expression levels of GST-P in the four treatment groups

Table II. *Effect of LTPs on body weight gain and liver weight during hepatic pre-cancerous lesion in rats.*

Groups	Final weight (g)	Liver weight (g)	Relative liver weight (g liver/100 g body weight)
Normal control	634.16±8.56	13.95±0.18	2.20±0.02
DEN/BP+0 mg/kg LTPs	632.88±10.63	22.17±0.40*	3.51±0.06*
DEN/BP+40 mg/kg LTPs	613.77±13.36#	21.62±0.56	3.54±0.07
DEN/BP+400 mg/kg LTPs	607.10±12.55#	20.12±0.52#	3.33±0.09#

Significance was determined by one-way analysis of variance. Data are mean±SE. *$p<0.05$ *vs*. Normal control group, #$p<0.05$ *vs*. LTP 0-mg/kg group.

Table III. *Fibrosis scoring in controls and LTPs groups.*

Groups	Score						
	0	1	2	3	4	5	6
Normal control (No.)	8	2	/	/	/	/	/
DEN/BP+0 mg/kg LTP (No.)	/	/	/	3	6	1	/
DEN/BP+40 mg/kg LTP (No.)	/	3	2	3	2	/	/
DEN/BP+400 mg/kg LTP (No.)	/	6	2	2	/	/	/

at 30 weeks post-treatment. As expected, GST-P was not expressed in normal control liver samples, as determined by immunohistochemical staining (Figure 6A). The GST-P-positive area and number became more evident in the DEN/PB-treated liver (Group 2, Figure 6B). Expression levels of GST-P were modified by the treatment within 40-mg/kg LTP (Group 3, Figure 6C), but this had no statistical significance. It was observed that the GST-P-positive area and number were significantly reduced by treatment with 400-mg/kg LTP compared to Group 2 (Figure 6D).

Effect of LTPs on hepatic cell proliferation. We compared the expression levels of anti-proliferating cell nuclear antigen (PCNA) in the liver between the four treatment groups (Figures 7 and 8). As expected, immunohistochemical analysis revealed that PCNA-positive cells were scarcely observed in the normal control liver (Figure 7A). After the DEN/PB treatment, PCNA-positive cells were significantly increased (Figure 7B). The number of PCNA-positive cells was significantly suppressed after the treatment with LTPs 40 or 400 mg/kg compared to group 2 (Figure 7C and D).

Effects of LTPs on DNA damage. Anti-8-hydroxy-2'-deoxuguanosine (8-OHdG) is used as a marker of oxidative DNA damage. We compared the expression levels of 8-OHdG in the liver between the four treatment groups (Figures 9 and 10). There was no positive immunolabelling of 8-OHdG observed in liver tissues from the normal control group (Figure 9A). However, after DEN/PB treatment, there

was a large number of cell which were positively labeled by 8-OHdG at their cytoplasm. Labeling with 8-OHdG was significantly suppressed after the treatment with LTPs at 40 or 400 mg/kg compared to group2 (Figure 9C and D).

Discussion

Although extensive studies have been conducted on the anti-carcinogenic properties of GTPs in several target organs (33), liver cancer preventive effect of LTPs as a dietary agent has not been investigated. The current study represents the first investigation of the chemo-preventive effect of LTPs against liver carcinogenesis induced by DBN/PB *in vivo*. It is well-known that DEN/PB causes the development of HCC through various stages with formation of pre-neoplastic foci, neoplastic nodules, and ultimately HCC nodules (34). DEN has been shown to induce tumors in rodents that closely-mimic a sub-class of human HCC (35).

In the present study, we analyzed the inhibitory effect of LTPs on the appearance of early hepatic pre-neoplastic events, employing a two-stage carcinogenic model combining DEN and PB. The findings of the present investigation demonstrated, for the first time, that LTPs inhibited the progression of liver carcinogenesis and prevented DBN/PB-induced hepatotoxicity.

Histological findings clearly showed that the normal architecture of hepatic tissue was damaged as a result of DENA/PB treatment. The hyperplastic nodular hepatocytes formed solid aggregates of mono- or multi-cellular thickness
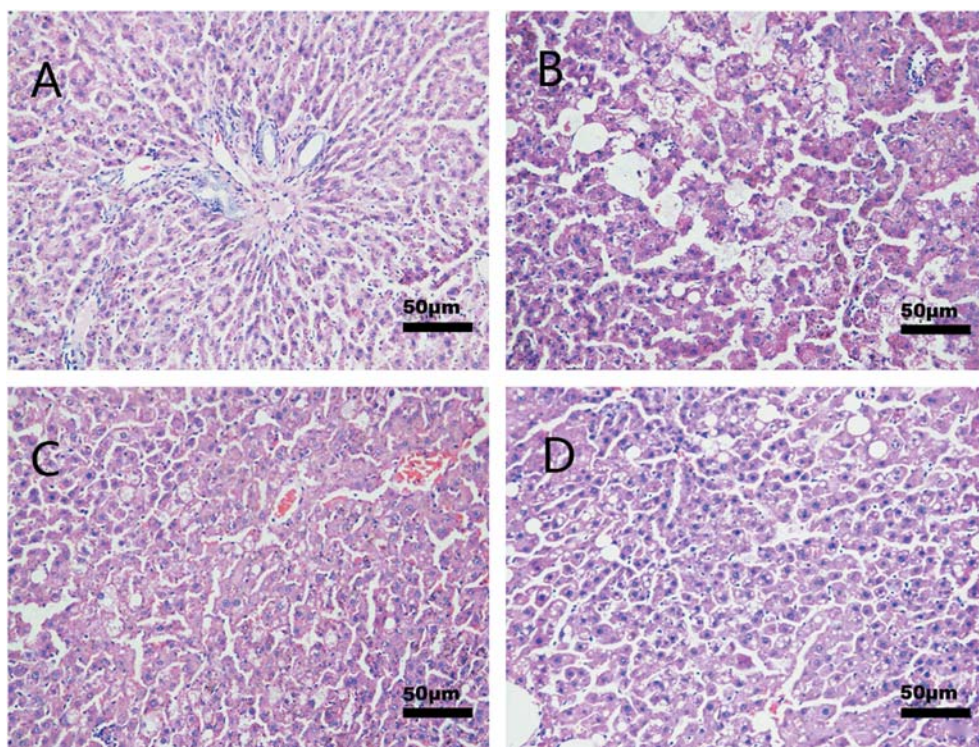
Figure 3. *Histological profile of representative liver tissue in LTPs-treated groups (H&E staining, original magnification, ×400). (A) Normal, (B) 0 mg/kg, (C) 40 mg/kg, (D) 400 mg/kg.*
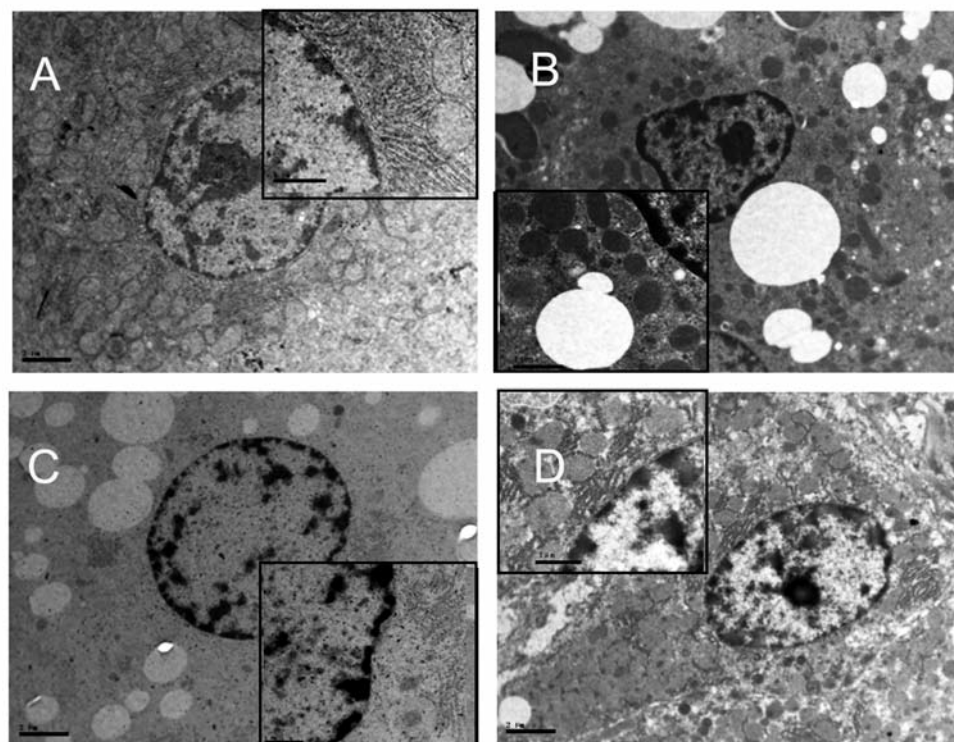

Figure 4. *Electron Microscope examination of rat liver tissue (TM, ×3700, ×8900), (A) Normal, (B) 0 mg/kg, (C) 40 mg/kg, (D) 400 mg/kg.*
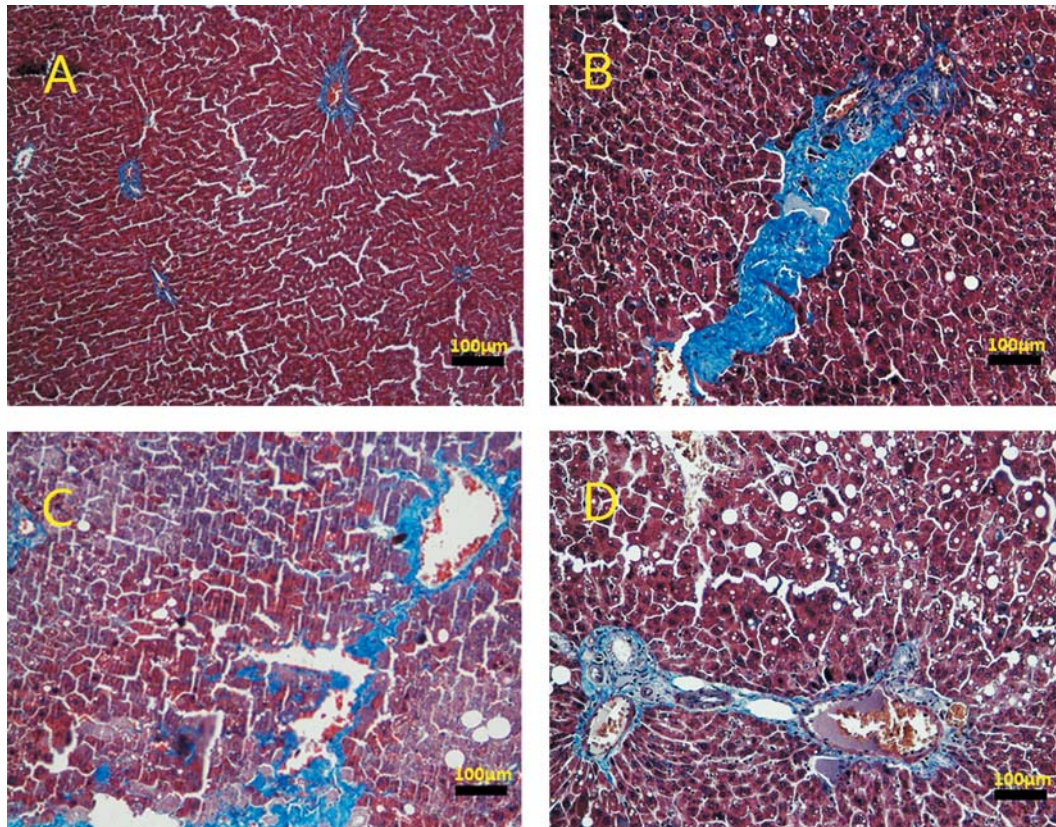
Figure 5. *Masson trichrome–stained histological slices excised from rat livers during the LTPs-treated groups (original magnification, ×200). (A) Normal control, (B) 0 mg/kg, (C) 40 mg/kg, (D) 400 mg/kg.*
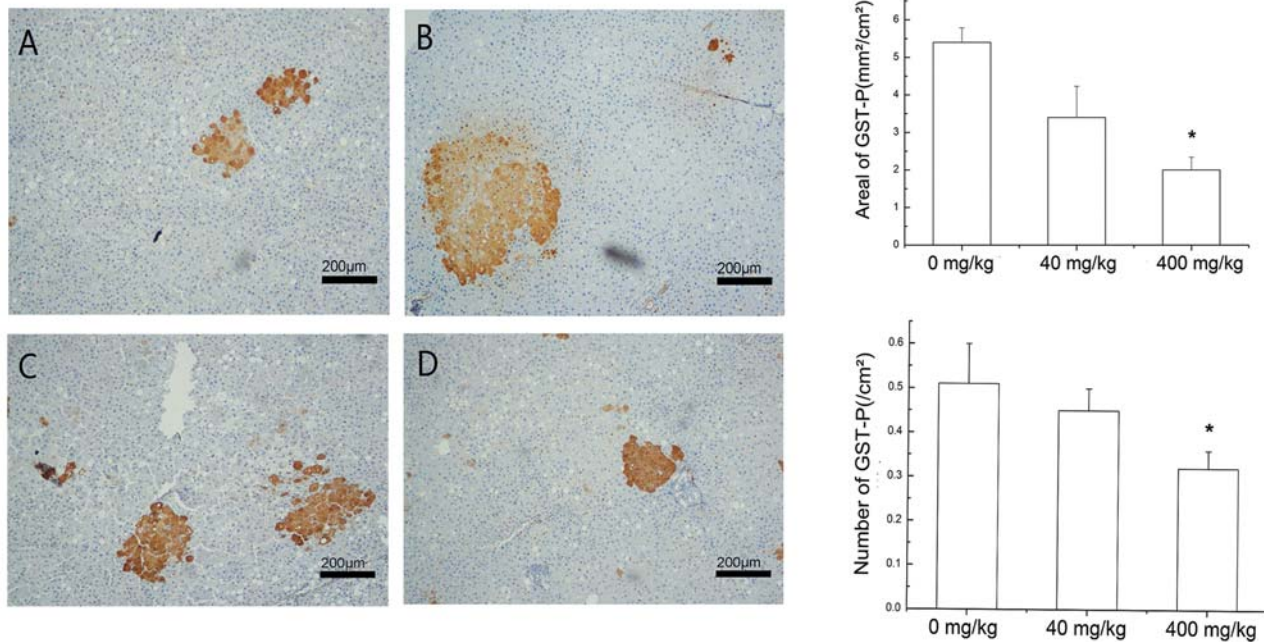

Figure 6. *LTPs inhibit DEN/PB induced expression of GST-P in rat liver. Quantitative analysis of GST-P-positive foci on GST-P-immunostained liver sections. The area of GST-P-positive foci was reduced in LTPs-treated rats. Significance was determined by one-way analysis of variance. Data are shown as mean±S.E. *p<0.05 vs. LTPs 0-mg/kg group.*
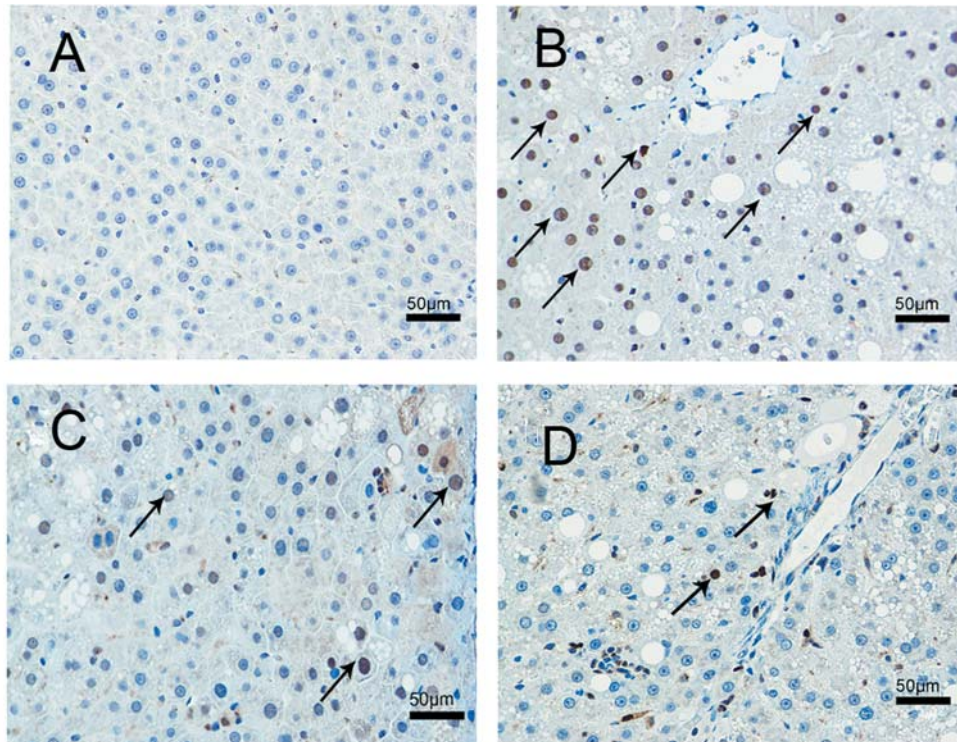
Figure 7. *Effects of LTPs on cell proliferation. Representative images of immunohistochemical staining with PCNA in liver section, from LTPs groups are shown (optical microscope, ×400). (A) Normal, (B) 0 mg/kg, (C) 40 mg/kg, (D) 400 mg/kg.*

with "hyperbasophilic foci" around the portal vein. These foci represent small pre-neoplastic focal lesions, leading to malignant transformation in later stages of carcinogenesis, with the formation of neoplastic nodules and ultimately HCC (36). In the DEN/PB-group, the majority of hepatocyte nodules consisted of a mixture of pre-neoplastic, neoplastic and diverse intermediate cells. Exposure to long-term LTP treatment elicited reduced hepatocyte aggregation and a reversal of heterogeneity towards normal cellular architecture (Figure 3). This LTP-mediated reduction in altered hepatocyte foci formation was closely-associated with a significant decrease in the number and area of GST-P positive foci. These foci are a reliable and sensitive marker of pre-neoplasia and neoplasia (37-38).

Liver fibrosis is a consequence of chronic liver injury from a variety of causes, including exposure to DEN. Liver fibrosis can lead to cirrhosis, liver failure, portal hypertension, and liver cancer (39). Liver fibrosis appears in the development of pre-cancerous lesions. We investigate the effect of LTPs during the progression of hepatic fibrosis. The result shows that 400 mg/kg LTPs could reduce the degree of fibrosis significantly (Figure 5D).

To investigate the mechanism by which LTPs attenuated hepatocarcinogenesis, we examined the extent of cell proliferation in DEN-induced tumorigenesis in the presence or absence of LTPs. Cellular proliferation is considered to play a
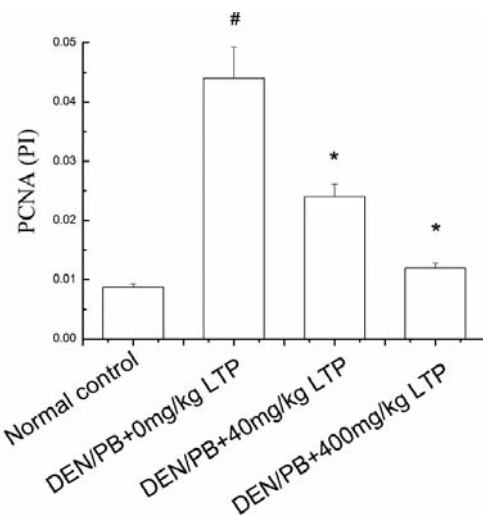

Figure 8. *Graphical representation of expression of PCNA labeling index. Data are mean±SE. #p<0.05 vs. Normal control group, *p<0.05 vs. the DEN/PB group.*

pivotal role in all phases of carcinogenesis with multiple genetic changes. PCNA is an essential regulator of the cell cycle, whose expression has been a useful tool to study cell proliferation (40).
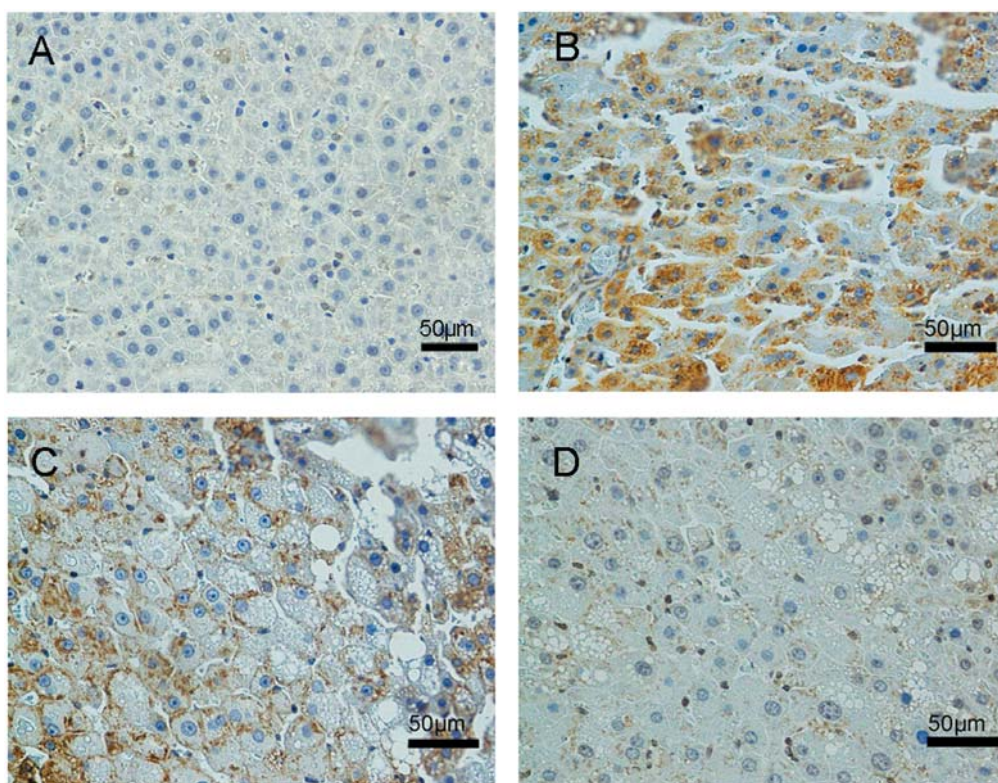
Figure 9. *Effects of LTP on DNA damage. Representative images of immunohistochemical staining with 8-OHdG in the liver section from LTPs-treated groups are given (optical microscope, ×400). (A) Normal, (B) 0 mg/kg, (C) 40 mg/kg, (D) 400 mg/kg.*

The PCNA protein has wider physiological functions in DNA replication, DNA repair and chromatin assembly; maximum expression of PCNA is thought to occur in the S phase (41). The expression PCNA is also present in non-proliferating tumor cells, as most tumors actively undergo DNA repair. The detection of PCNA by immunohistochemical technique is a common method for the study of proliferative activity in transformed cells (42). Increased cell proliferation and DNA damage mechanisms are two of the physiological alterations that cancer cells undergo as they continue to grow. The increased number of PCNA-positive hepatocytes in Group 2 compared to the Normal group is regarded as proliferating cells especially in S-phase (Figure 7B). These results suggest that DEN/PB has a positive effect on hepatocyte proliferation and tumor promoting potential in the livers of treated rats. LTPs treatment was accompanied by a remarkable reduction in the number of PCNA positive cells, indicating that it is capable of suppressing malignant proliferation of hepatocytes in experimental hepatocarcinogenesis through its anti-proliferative activity.

The presence of 8-OHdG is a well-known marker of oxidative damage in cellular components (43). In the present study, 8-OHdG levels significantly increased in Group 2 (Figure 9B). There are more than 100 types of oxidative base modification in mammalian DNA (44), and 8-hydroxy-2-
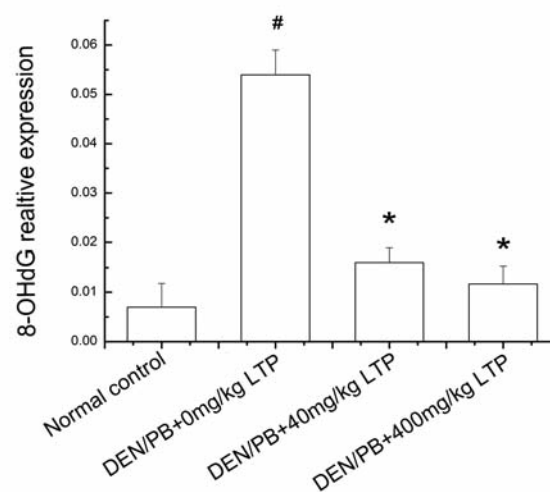

Figure 10. *Effects of two kinds of tea polyphenols on 8-OHdG relative expression. Data are mean±S.E. $^{\#}p<0.05$ vs. Normal control group, $^{*}p<0.05$ vs. the DEN/PB group.*

deoxyguanosine (8-OHdG) is one of the most abundant types of oxidative damage to DNA (45). Cellular nuclei are stained positive for the presence of 8-OHdG in most of this study's

immunohistochemical results (46-47). Researchers have observed the evidence of damage to mitochondrial DNA damage in the cytoplasm (48). Immunohistochemical and electron microscopy were both positive and revealed serious mitochondrial damage (Figures 3 and 4). We speculate that the brown coloration of the mitochondria-rich, liver cells could be the result of oxidative damage to hepatocytes' mitochondrial DNA. The results of our study suggest that 8-OHdG production resulting from the Reactive Oxygen Species (ROS) generation may result in enhanced induction of pre-neoplastic lesions in the liver of rats given DEN/PB. Generation of ROS is thought to have a bilateral character; one being to damage the cell component, and the other being to enhance the proliferation of cells (49).

In the current study, PCNA and 8-OHdG staining were increased in a two-stage model of hepatocarcinogenesis in Sprague Dawley rats, and suggested that ROS generation enhanced tumor promotion. The ability of 40-mg/kg and 400-mg/kg LTPs to reduce the number of proliferative and DNA damaged cells has been implicated in the chemo-preventive action of LTPs against DEN/PB-initiated rat hepatocellular carcinogenesis.

In summary, the data presented here show that LTPs dramatically inhibited foci of altered hepatocytes (FAH) formation in livers of DEN/PB treated rats. This inhibition was associated with reduced cell proliferation, decreased fibrosis, and the reduction of markers for oxidative damage to DNA. It is supposed that LTPs has higher bioavailability by oral administration compared with GTPs. Although the delivery route of LTPs into the human body is still not clear, it is postulated to be performed *via* the chylomicron pathway (17). In this case, LTPs could be associated with lipoprotein particles, which significantly reduce potential binding with serum proteins. This would increase the level of LTPs in lipoproteins such as LDL, prior to internalization by hepatocytes (17). We speculate that the anticancer benefits of LTPs could be superior to those of GTPs, as a result of their improved bioavailability. Further investigations are currently underway to study the mechanism of action of lipid soluble tea polyphenols in greater detail.

Acknowledgements

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