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Original article

HEPATOPROTECTIVE EFFECT OF CAFFEINE ON DIETHYLNITROSAMINE-INDUCED LIVER INJURY IN RATS

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Summary

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In the current study, we investigated the protective effects of the caffeine (Caff) on hepatic damage induced by diethylnitrosamine (DEN) administration in rats. Animals were divided into four groups with 10 animals in each group. Animals in group 1 were untreated (control). Rats in group 2 were injected with a single dose of 200 mg/kg DEN, intraperitoneally (DEN group), those from group 3 were intraperitoneally injected with 100 mg/kg caffeine daily for four weeks (Caff group) and rats from group 4 (DEN+Caff) received the same DEN dose as group 2 and daily caffeine treatments as group 3. After 4 weeks, blood was collected for analysis of activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Liver specimens were taken for histopathological examination. The single intraperitoneal administration of 200 mg/kg DEN to rats resulted in significantly elevated levels of serum AST, ALT and ALP indicative of hepatocellular damage. Histopathological examination revealed proliferation of stellate cells, necrosis, cell swelling and karyomegaly in DEN and DEN+Caff groups, with lower intensity in the DEN+Caff group. The results from the present study suggested that caffeine exhibited hepatoprotective properties against diethylnitrosamine-induced hepatocellular damage in rats.

Key words: caffeine, diethylnitrosamine, liver, rat, toxicity

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in many popular beverages, including cocoa, tea and coffee. It is also widely used medically as CNS, respiratory and cardiac stimulant, smooth muscle relaxant, analgesic and diuretic (Serafin, 1996). Epidemiological studies have shown that caffeine con-

sumption reduced increased serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are markers of liver injury, and decreased the risk of chronic liver disease (Freedman *et al.,* 2009). However, the major cellular and molecular mechanisms of the hepatoprotective action of caffeine are poorly defined (Okano *et al.,* 2008). Some studies attributed the hepatoprotective effect of caffeine in alcohol liver injury to its anti-inflammatory and antioxidant effects). Observations based on chemical studies showed that caffeine was able to scavenge reactive oxygen species (ROS), particularly the hydroxyl radical (OH·), known to be generated in the body by irradiation with various electromagnetic frequencies such as exposure to UV, as well as by many ambient physiologic reactions involving oxygen utilisation (Shi *et al.,* 1991; Stadler & Fay, 1995). Furthermore, it was reported that caffeine decreased tissue lipid peroxidation, protected membranes from ROS damage and improved oxidative stress control (Demirts *et al.,* 2012).

In food processing, nitrites are added to smoked fish, pickled vegetables and cured meats to inhibit bacterial growth and as colorants and flavour enhancers. In this process, some nitrites are converted to nitrosamines due to the effect of heat and gastric acid, making these foods the major dietary source of nitrosamines (Hill, 1988; Thirunavukkarasu & Sakthisekaran, 2003). Nitrosamines are amongst the most potent toxins. N-nitrosodiethylamine (DEN) is an N-nitroso alkyl compound described as an effective hepatotoxin in experimental animals, producing toxicity after repeated administration (Jose *et al*., 1998). DEN is found in a wide variety of foods such as cheese, soybeans, smoked, salted and dried fish, cured meat and alcoholic beverages (Liao *et al.,* 2001). It is reported to be metabolically activated by cytochrome P450 enzymes to form reactive electrophiles which induce oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Archer, 1989). Oxidative stress is considered essential to DEN-induced hepatotoxicity and the use of antioxidant agents reduced liver damage (Vitaglione *et al.,* 2004; Pradeep *et al.,* 2007). Various plants and plant derived products have been tested and found to be effective against DEN-induced hepatocarcinogenesis and hepatotoxicity (Poojari *et al.*, 2010; Pradeep *et al.*, 2010; Zhang *et al.*, 2012). Lee *et al.* (2007) reported that caffeine had a protective effect against liver injury induced by carbon tetrachloride.

In the light of these observations, it was decided to evaluate the efficacy of caffeine against diethylnitrosamine induced hepatocellular damage. Thus, the aim of the present study was to examine the antioxidant and protective effects of caffeine on hepatotoxicity induced by diethylnitrosamine in rats.

MATERIALS AND METHODS

Animals and treatments

The study was approved by the Animal Ethics Committee of the University of Shahid Chamran University, Iran. Forty female albino Wistar rats weighing 180±5 g were kept in the laboratory under constant temperature (24±2 ^{o}C) for at least one week before and through the experiment. The animals were fed a standard diet and water, available *ad libitum* and maintained in accordance with the guidelines prescribed by the Faculty of Science.

The experimental rats were divided into four groups with 10 animals in each group:

- Group I (control): Animals were fed on the standard diet and served as control group.
- Group II (DEN): Rats were injected intraperitoneally with a single dose of diethylnitrosamine at 200 mg/kg (Sigma Aldrich, USA) (Shaarawy *et al.,* 2009).
- Group III (Caff): Animals were intraperitoneally injected with a daily dose of 100 mg/kg caffeine (Sigma Aldrich, USA) for four weeks.
- Group IV(DEN+Caff): Rats were injected with DEN dose as group 2 and received daily caffeine treatments as group 3.

Biochemical assays

The treated and control animals were sacrificed by decapitation after 4 weeks of treatment. For biochemical study, sera were obtained by centrifugation of the blood samples and stored at –20 ^{o}C until assayed. The serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined using commercially available kits (Pars Azmoon, Tehran, Iran).

Histopathological examinations

After sacrifice, the livers of rats were removed and fixed in 10% neutral formalin. Fixed materials were embedded in paraffin and sections of 5 µm thickness were cut. Slides were stained with haematoxylin and eosin for histopathological examination.

Statistical analysis

The results were expressed as mean ± SEM of different groups. The differences between the mean values were evaluated by ANOVA followed by Tukey-Kramer test. A value of $P<0.05$ was accepted as significant.

RESULTS

Biochemical results

Table 1 shows serum ALT, AST and ALP concentrations in control and experimental animals. Diethylnitrosamine (Group II) induced hepatotoxicity is shown by a 2-fold increase in the activity of AST and a 3-fold increase in ALT and ALP in the serum of rats as compared to controls (Group I). This increased activity of studied liver enzymes after DEN challenge was significantly decreased on week 4 after the treatment with caffeine (Group IV).

Histopathological results

The histopathological examination of the liver in control or caffeine-treated rats

Table 1. Effect of caffeine on serum alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels in serum during diethylnitrosamine induced hepatotoxicity. Results are given as mean±SEM (n=10). Group I: untreated rats; Group II: treated with a single dose of 200 mg/kg DEN treated rats; Group III: treated with daily dose of 100 mg/kg caffeine over 4 weeks; Group IV: treated with DEN and caffeine at the same time.

Liver enzymes	Group 1 (control)	Group II (DEN)	Group III (Caff)	Group IV (DEN+Caff)
ALT	53 ± 9	148 ± 18[a]	61 ± 12	74 ±13
ALP	46 ± 7	122 ± 16[a]	54 ± 9	63 ± 11
AST	73 ± 11	150 ± 19[a]	70 ± 13	100 ± 14

[a] $P<0.05$ compared to control group.

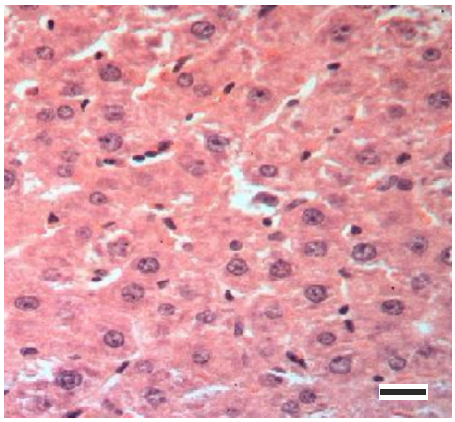

Fig. 1. Photomicrograph of liver specimens from control and caffeine-treated rats showing normal liver histology with unremarkable central vein. H & E, bar=20 μm.

revealed entirely normal histological features (Fig. 1).

The administration of DEN caused significant histological damage to the liver. Examination of the liver sections of animals after treatment with DEN for 4 weeks, demonstrated cell swelling and single hepatocellular necrosis (Fig. 2A). Dysplastic hepatocytes were observed with enlarged nuclei (karyomegaly) and multiple nucleoli in the liver sections of rats treated with DEN (Fig. 2B). There were marked proliferation of hepatic stellate cells (HSCs) in the portal area as well as focal HSCs proliferation (Fig. 2C).

Treating DEN-challenged animals with caffeine revealed an improvement in the histopathological appearance of the liver. Most of the hepatocytes appeared normal although few cells were damaged (Fig. 3).

A comparison of changes in liver structures of different groups is presented in Table 2.

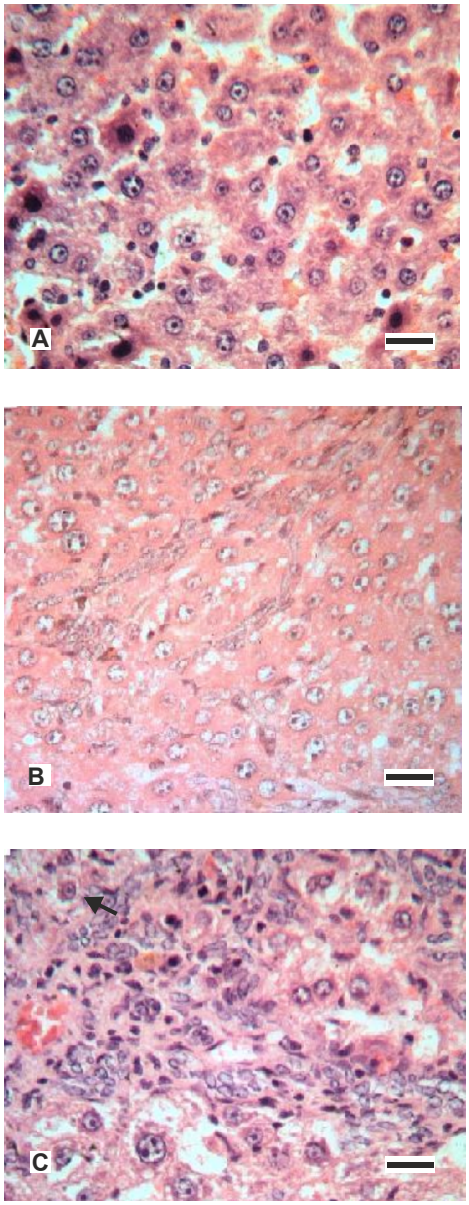

Fig. 2. Photomicrograph of liver from rat treated with DEN showing central vein surrounded by extensive necrosis (A), karyomegaly (B) and proliferation of hepatic stellate cells (C). H & E, bar=20 μm.

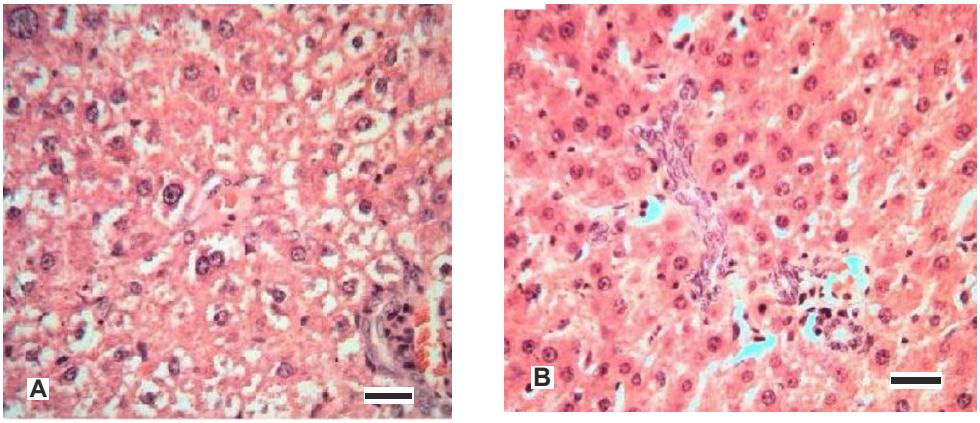

Fig. 3. Photomicrographs of liver specimens from rats treated with DEN + coffeine showing mild necrosis and mild karyomegaly (A) and mild proliferation of hepatic stellate cells (B). H & E, bar=20 μm.

Table 2. Quantitative assessment of renal histological changes in rats with diethylnitrosamine induced hepatotoxicity. Group I: untreated rats; Group II: treated with a single dose of 200 mg/kg DEN treated rats; Group III: treated with daily dose of 100 mg/kg caffeine over 4 weeks; Group IV: treated with DEN and Caff at the same time.

Groups	Karyo-megaly	Cell swelling	Hepatocyte necrosis	Proliferation of hepatic stellate cells (HSCs)
Group I (control)	–	–	–	–
Group II (DEN)	+++	+++	+++	+++
Group III (Caff)	–	–	–	–
Group IV (DEN+Caff)	++	++	+	+

(–): no histological damage was observed, (+): 25% of hepatocytes showed histological damage, (++): 25% to 75% of hepatocytes showed histological damage, (+++): over 75% of hepatocytes showed histological damage.

DISCUSSION

Diethylnitrosamine, one of the most important environmental carcinogens, has been suggested to generate ROS resulting in oxidative stress and cellular injury (Bartsch *at el.,* 1989). After being metabolised by cytochrome p450, DEN generates highly reactive free radicals and initiates lipid peroxidation of the cell membrane of the endoplasmic reticulum and causes a chain reaction. Produced ROS can cause oxidative damage in DNA, proteins and lipids (Archer, 1989; Vitaglione *et al.,* 2004). Since the liver is the main site of DEN metabolism, the production of ROS in the liver may be responsible for its carcinogenic effects (Bansal *et al.,* 2000).

The present study documents the hepatoprotective effect of caffeine against liver injury induced by DEN in rats. In this study, DEN administration to rats led to marked elevation in serum levels of ALT, AST and ALP indicating hepatocellular damage as previously reported. This might be due to the possible release of these enzymes from the cytoplasm into the

blood circulation rapidly after rupture of the plasma membrane and cellular damage (Bansal *et al.,* 2000). Serum AST, ALT and ALP are the most sensitive markers employed in the diagnosis of hepatic damage (Naik & Panda, 2007). The increase in the activities of these enzymes in serum and subsequent fall in the tissue might be due to the leakage of these cytosolic enzymes into the circulatory system resulting from hepatocellular damage after DEN administration. Several studies have reported similar elevation in the activities of serum AST, ALT, and ALP during diethylnitrosamine administration (Shaarawy *et al.,* 2009; Poojari *et al.,* 2010).

Treatment with caffeine significantly reduced the activities of the above marker enzymes in DEN-treated rats. This indicates that caffeine tends to prevent liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes through membranes, exhibiting hepatoprotective activity. This might be the reason for the restoration in the activities of the marker enzymes during administration of caffeine.

The biochemical findings are supported by histopathological findings in liver specimens. Microscopic results in DEN group showed different lesions including necrosis, degeneration, karyomegaly and proliferation of hepatic stellate cell (HSCs) in agreement with other researches (Shaarawy *et al.,* 2009; Choi *et al.,* 2010). Transactivation of quiescent stellate cells contributes to hepatic fibrosis (Toyoki *et al.,* 1998). Activated HSCs are associated with cell proliferation and the accumulation of extracellular matrix proteins, including α-SMA and collagen type I and III. Furthermore, α-SMA is a marker for the early stage of hepatic fibrosis (Pinzani *et al.,* 1998; Pinzan & Marra, 2001). In contrast to this, liver sections of DEN+Coff group showed improved hepatocellular architecture with signs of recovery and decreased HSCs proliferation; however moderate cell swelling was obvious.

Caffeine is rich in phytochemical derivatives such as triterpenes, flavonoids or polyphenols. Many studies reported that the preventive effects of caffeine could be attributed to their protective activity (Cavin *et al.,* 2001). Huber *et al.* (2002) reported that kahweol and cafestol phenolic diterpenes of caffeine inhibit lipid peroxidation.

In conclusion, our present investigation showed that caffeine had excellent hepatoprotective properties manifested by restoring the normal serum concentrations of hepatic marker enzymes and liver histopathological findings after diethylnitrosamine induced liver injury in rats.

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