

The effects of stobadine on purine metabolism in rats treated with carbon tetrachloride

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Aim: The aim of the present study was to investigate the relationship between liver damage induced by carbon tetrachloride (CCl₄) and adenosine deaminase (ADA), 5'-nucleotidase (5'NT), xanthine oxidase (XO) enzyme activities, and malondialdehyde (MDA) levels. The effect of stobadine on purine metabolism was also evaluated.

Materials and methods: This study measured the effect of CCl₄ on ADA, 5'NT, XO enzyme activities, and MDA levels, and measured the histopathologic changes in liver cells.

Results: Our research found that ADA, XO activities, and MDA levels increased in the CCl₄ group when compared with the control group. This was also supported by the histopathological changes in the CCl₄ and CCl₄+stobadine group found when compared with the control group. Stobadine could not protect cells against CCl₄ damage.

Conclusion: The present study helps explain the biochemical mechanisms of liver injury formed by CCl₄ and shows the relationship between the purine degradation pathway and CCl₄-induced cellular toxic effect. Cellular damage is dependent upon the biochemical response to increased purine degradation pathway enzyme activities, which also may be responsible for decreasing liver cellular adenosine levels and S-adenosyl-methionine and increasing superoxide anion and hydrogen peroxide levels. It is suggested that the inhibition of key enzymes of the purine degradation pathway (particularly ADA and XO) may prevent liver damage.

Key words: CCl₄, adenosine deaminase, 5'-nucleotidase, xanthine oxidase, malondialdehyde, S-adenosyl-methionine

Introduction

Exposure to high concentrations of carbon tetrachloride (CCl₄) degenerates the liver (1). Experiments have been carried out to generate a CCl₄-induced cirrhosis model using experimental animals because of CCl₄'s selective hepatotoxic effect (2). Past studies have shown that chronic exposure to CCl₄ can induce cirrhosis by causing the production of free radicals (3).

After the bioactivation of CCl₄, the unstable free radical molecule trichloromethyl (CCl₃·) is created by hepatic microsomal cytochromes P450. CCl₃· reacts very rapidly with O₂ to yield CCl₃O₂· (4). Both CCl₃· and CCl₃O₂· can form secondary structures such as conjugated dienes, lipid hydroperoxides, and malondialdehyde (MDA) (5). Both these free radicals and lipid peroxidation injure the hepatocytes and lead to necrosis (6).

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Degradation of purines and their nucleotides occurs during the turnover of endogenous nucleic acids and the degradation of ingested nucleic acids. 5'-Nucleotidase (5'NT), adenosine deaminase (ADA), and xanthine oxidase (XO) are key enzymes of the purine nucleotide degradation pathway (7,8). 5'NT has an important function in the protection of the intracellular nucleotide pool because it regulates levels of adenosine 5'-monophosphate (5'AMP) and guanosine 5'-monophosphate (5'GMP), both of which are main substrates for DNA metabolism (8). 5'NT deficiency can cause many pathological conditions such as spherocytic anemia and immune system defects (9).

ADA is a key enzyme involved in the regulation of the concentration of both intracellular and extracellular adenosine and deoxyadenosine (10). Increased ATP and deoxyATP nucleotide levels cause the inhibition of the ribonucleotide reductase enzyme, which is responsible for the regulation of the deoxy form of nucleotides in cells (11). Adenosine, the other substrate of ADA, has a key role in S-adenosyl-methionine (SAM) synthesis. SAM is critical for transmethylation and transsulfuration reactions and glutathione synthesis (12). SAM is also perceived as a precursor antioxidant molecule and as a source of methyl groups for methylation reactions. It is synthesized in the presence of methionine plus adenine (12,13). SAM has been shown to be beneficial for alcoholic liver disease (14). Increased ADA activity leads to the decrease of adenosine and deoxyadenosine and results in the inhibition of SAM synthesis (15).

ADA degrades adenosine-producing inosine. Inosine is used by the purine nucleoside phosphorylase (PNP) enzyme and is turned to hypoxanthine. XO converts inosine to xanthine. XO is the most important cellular source of enzymatic radicals. XO leads to the formation of oxygen radicals and hydrogen peroxide during the 2 steps of hypoxanthine and xanthine utilization (16,17). The structural change of xanthine dehydrogenase (XD) to XO carries weight in the production of enzymatic-based free radical damage (17). Sakuma et al. suggested that monochloramine (NH_2Cl) has the potential to convert XD into XO in the liver, which in turn may induce reactive oxygen species (ROS) generation in the cell (18).

New antioxidants with different mechanisms have been developed to prevent the formation of ROS or to reduce their harmful effects, and stobadine (ST; (-)-cis-2,8-dimethyl-2,3,4,4a,5,9b-hexa-hydro-1H-pyrido[4,3-b]indole) is one of these antioxidants (19). Stobadine has been shown to be able to scavenge hydroxyl, alkoxyl, and peroxy radicals and prevent superoxide radical generation. The antioxidant properties of stobadine are conditioned mainly by its indole ring compounds' ability to stabilize radicals. Thus, it reduces oxidative stress-induced lipid peroxidation and protein oxidation (20,21).

In the present study, we examined the relationship between CCl_4 toxicity and purine degradation and salvage pathway enzymes, which are responsible for adenosine degradation and free radicals production. We also investigated the protective role of stobadine on this metabolism.

Materials and methods

This study was approved by the Experimental Ethics Committee of Gazi University School of Medicine, Ankara, Turkey, and the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* was followed.

Surgery and experimental protocol

In the present study, we used 40 Wistar albino (250-300 g) rats that were housed in wire bottom cages, given a free diet, and exposed to a 12-h light/dark cycle. They were randomly assigned into 4 groups containing 10 rats each. The groups were as follows: group 1 was the control, group 2 was given stobadine, group 3 was given CCl_4 , and group 4 was given CCl_4 +stobadine. The rats in group 2 were given 24.7 mg/kg of stobadine daily dissolved in a 0.5% Avicel (carboxymethyl cellulose) solution via orogastric tube 3 times per week for 8 weeks (22,23). Group 3 rats were given CCl_4 dissolved in olive oil at a ratio of 1:10 (v/v) intraperitoneally 3 times a week at levels of 0.3 mL/kg in the first week, 0.7 mL/kg in the second week, and 1.0 mL/kg in the last 6 weeks for a total of 8 weeks. The rats in group 4 were given 24.7 mg/kg of stobadine daily dissolved in a 0.5% Avicel (carboxymethyl cellulose) solution via orogastric tube, and also CCl_4 dissolved in olive oil at a ratio of 1:10 (v/v) intraperitoneally 3 times per week at

levels of 0.3 mL/kg in the first week, 0.7 mL/kg in the second week, and 1.0 mL/kg in the last 6 weeks for a total of 8 weeks (24).

After 24 h had elapsed after the last administration of drugs, all rats were sacrificed under general anesthesia and muscle relaxant (40 mg/kg Alfamin and 2.5 mg/kg Alfazyme intramuscularly). Next, their livers were harvested and cleaned with a 0.9% NaCl solution. The liver tissue samples were stored in a 10% formalin solution for histopathology analysis. The remaining liver tissues were immediately frozen in liquid nitrogen and stored in a freezer at -80°C until all measurements were complete.

Tissue preparation and analysis of liver enzyme activities

The frozen liver tissues were homogenized in a 0.9% NaCl solution. The homogenates were centrifuged at $16000 \times g$ at 4°C for 20 min. The protein content of supernatants was measured according to the method described by Lowry (25). 5'NT, ADA, and XO enzyme activities in the liver tissues were also measured according to the methods described by Donald et al., Guisti et al., and Hashimoto et al., respectively (26-28). All enzyme activities are presented as mIU/mg protein. MDA levels were measured according to the method described by Van Ye et al. (29). Results are presented as μmol MDA/mg protein.

Histopathological evaluation

We used the Bio-Optica aniline blue staining method applied to Masson's trichrome stain in the histopathological examination. (Bio-Optica Masson's

trichrome, Cat. No. 04-010802, Milan, Italy).

In addition, tissues were evaluated by the immunohistochemical method. For this, the TGF- β_3 antibody was used to examine the immunohistochemical staining. A Leica DM 4000 B microscope was used to evaluate liver fibrosis.

Statistical analysis

We used SPSS 15.0 for Windows and Microsoft Excel for Windows XP to perform the statistical analyses and create graphs. Data were presented as the mean \pm standard deviation ($X \pm \text{SD}$). The Mann-Whitney U test and Kruskal-Wallis variance analysis were used for the statistical analyses. $P < 0.01$ was considered to be significant.

Results

Our results are given in the Table. ADA and XO activities were significantly increased in the CCl₄ and CCl₄+stobadine groups compared with the control and stobadine groups. Stobadine alone could not approximate the activities of these 2 enzymes in the control group or in the CCl₄+stobadine group. ADA and XO enzyme activities did not differ in the CCl₄ group as compared with the CCl₄+stobadine group. When stobadine was introduced in the CCl₄+stobadine group, the 5'NT enzyme activity rose to that of the levels of the control group. MDA levels significantly increased in the CCl₄ and CCl₄+stobadine groups when compared with the control group and the stobadine group. MDA levels

Table. 5'NT, ADA, and XO enzyme activities and MDA levels in rat liver tissue.

Groups (n = 10)	5'NT (mIU/mg prot.)	ADA (mIU/mg prot.)	XO (mIU/mg prot.)	MDA (μmol /mg prot.)
Control	756.2 \pm 116.1	20.8 \pm 2.1	321.2 \pm 112.1	10.4 \pm 0.9
Stobadine	1171.7 \pm 164.3 ^a	23.6 \pm 2.5	344.11 \pm 103.3	10.1 \pm 1.0
CCl ₄	714.1 \pm 109.2 ^b	220.9 \pm 29.9 ^{a,b}	821.11 \pm 84.2 ^{a,b}	22.3 \pm 2.5 ^{a,b}
CCl ₄ +stobadine	951.5 \pm 188.7 ^{a,c}	253.3 \pm 23.4 ^{a,b}	798.8 \pm 72.3 ^{a,b}	17.5 \pm 1.6 ^{a,b,c}

^aP < 0.01, compared with control group

^bP < 0.01, compared with stobadine group

^cP < 0.01, compared with CCl₄ group

were decreased in the CCl_4 +stobadine group as compared with the CCl_4 group.

Our histopathological results show that the portal vein, branches of the hepatic artery, and the bile duct around the arrangement of collagen fibers were normal in the control group (Figure 1). Parenchymal liver cells, sinusoids, and vascular structures close to those of the control group were noticed in the stobadine group (Figure 2). Developing advanced oil necrosis, connective tissue cells leading to fibrosis, and excessive collagen fibers were identified in the CCl_4 group (Figure 3). Less cellular necrosis and fatty vacuole formation was seen in the CCl_4 +stobadine

group. However, continued signs of fibrosis were observed in this group (Figure 4). In our study, the immunohistochemical method of examining stained liver tissue sections was also used. In the control group, $\text{TGF-}\beta_3$ immunoreactivity was found to be weak (Figure 5). Histopathologic results were similarly obtained in the stobadine group when compared with the control group (Figure 6). $\text{TGF-}\beta_3$ immune reactivity significantly increased in the liver parenchymal cells and the septal structures in the CCl_4 group (Figure 7). Moderate immunoreactivity of $\text{TGF-}\beta_3$ was viewed in the CCl_4 +stobadine group (Figure 8).

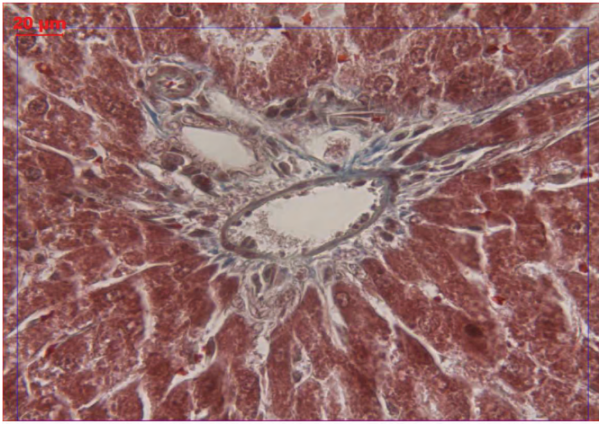


Figure 1. Control group: portal vein, branches of hepatic artery, and bile duct around the arrangement of the collagen fibers was normal (Masson's trichrome, 400 \times).

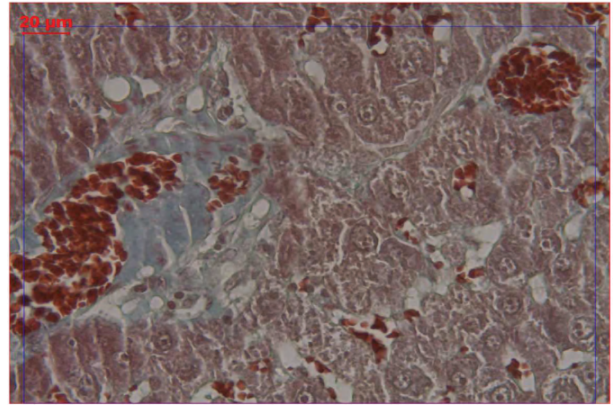


Figure 2. Stobadine group: parenchymal liver cells, sinusoids, and vascular structures close to the control group were noticed (Masson's trichrome, 400 \times).

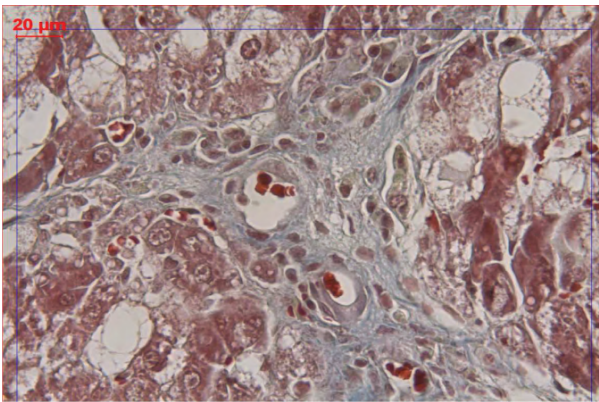


Figure 3. CCl_4 group: developing advanced oil necrosis, connective tissue cells leading to fibrosis, and excessive collagen fibers were identified (Masson's trichrome, 400 \times).

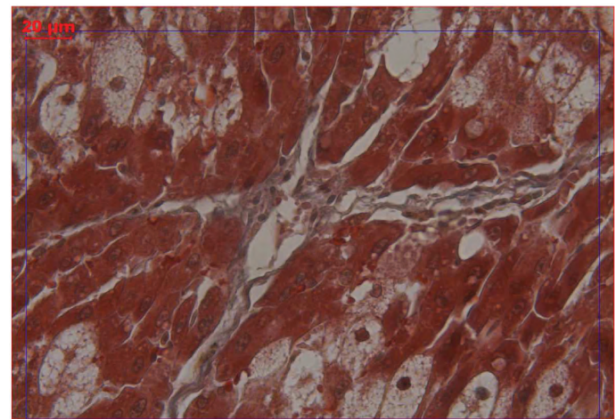


Figure 4. CCl_4 +stobadine group: less cellular necrosis and fatty vacuole formation was seen, but continued signs of fibrosis were observed (Masson's trichrome, 400 \times).

Discussion

In our study, enzyme activities of the purine salvage pathway were evaluated in rats administered with CCl₄. The amount of liver damage was further supported by the results of the histopathological examination of liver tissues stained with Masson's trichrome and by using the immunohistochemical method. The changes in enzyme activities that may arise with CCl₄-induced damage will help us to understand metabolism and produce solutions for the problems that cause liver damage. In addition, we investigated the effects of the new antioxidant molecule stobadine on the purine salvage pathway, which contains the most important enzymatic step of free radical generation.

The most common chemical agent used to generate liver damage is CCl₄. It is widely used by researchers who seek to understand the biochemical basis of liver necrosis and fibrosis. Free radicals resulting from the effects of toxins cause unsaturated fatty acid peroxidation of cellular membrane phospholipids that then lead to cellular necrosis (3-6). Many antioxidant molecules are used in order to prevent CCl₄ toxicity. Stobadine prevents oxidation of -SH groups and amino acids, and the formation of single oxygen molecules. Stobadine can also neutralize hydroxyl, peroxy, and alkoxy radicals (20,21).

Purine metabolism is important in providing a substrate for SAM, which is perceived as a precursor antioxidant molecule and a source of methyl groups for methylation reactions (13,14). Studies show that

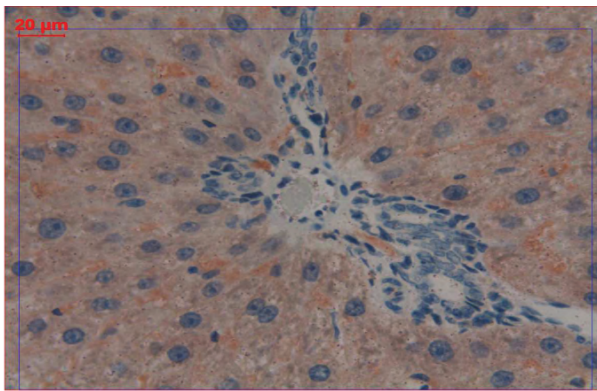


Figure 5. Control group: TGF-β₃ immunoreactivity was found to be weak (anti-TGF-β₃, 400×).

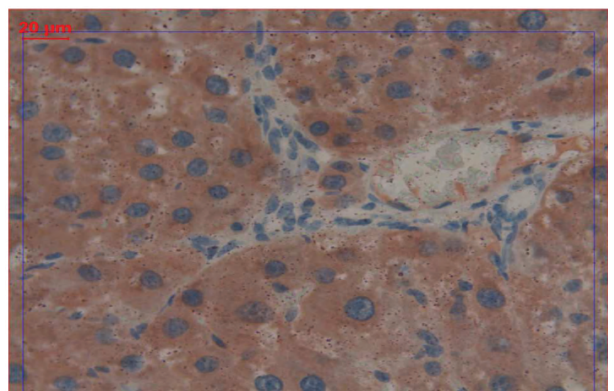


Figure 6. Stobadine group: histopathologic results were similar to the control group (anti-TGF-β₃, 400×).

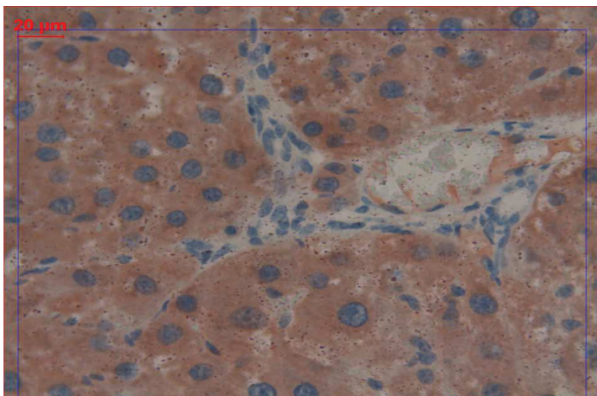


Figure 7. CCl₄ group: TGF-β₃ immunoreactivity was significantly increased in the liver parenchymal cells and the septal structures (anti-TGF-β₃, 400×).

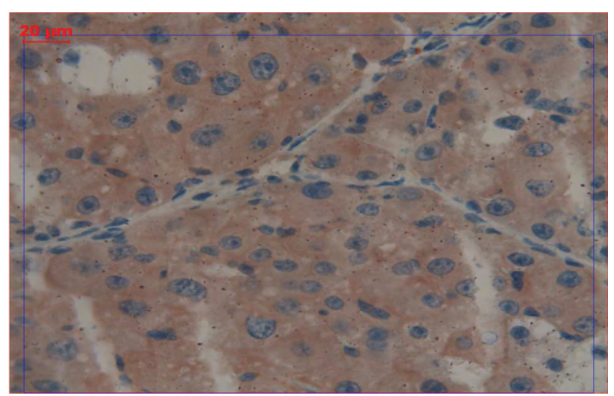


Figure 8. CCl₄+stobadine group: moderate immunoreactivity of TGF-β₃ was seen (anti-TGF-β₃, 400×).

SAM protects against CCl_4 -induced liver damage and alcohol toxicity (30,31). XO catalyses the final enzymatic steps of the purine degradation pathway and is responsible for cellular damage through the production of oxygen radicals and hydrogen peroxide. XO reactions are enzymatically the most important oxygen radical producers in cells. It has been suggested that NH_2Cl has the potential to convert XD into XO in the liver, which in turn may induce ROS generation in cells (18).

In our study, it is important to note that CCl_4 increased ADA and XO activities yet had no effect on 5'NT activity. We suggest that the metabolic result of this finding is reduced levels of adenosine and the increase in oxygen radicals and hydrogen peroxide. Increased adenosine destruction led to the increase in the substrate for XO and thus an increase in oxygen-based radicals. Histopathologic results of the damage caused by CCl_4 were fibrosis and necrosis of the liver tissue, which were determined via Masson's trichrome and the immunohistochemical method. These findings are important in defining cellular damage caused by CCl_4 . Increased MDA levels detected in the CCl_4 and CCl_4 +stobadine groups as compared with the other groups support the lipid peroxidation and histopathologic results that showed lipid membrane damage.

Increased ADA activity results in the reduction of SAM metabolism and an increase in oxygen-based free radicals. These 2 harmful effects are thought to be mainly responsible for the damage caused by the use of CCl_4 . The histopathologic demonstration of ongoing damage in the CCl_4 +stobadine group led to this hypothesis. Despite the partial adjustment of cellular damage by stobadine during the use of CCl_4 , cellular damage was still found. This shows that stobadine has a limited protective effect and does not prevent lipid peroxidation. Lower MDA levels in the CCl_4 +stobadine group than the CCl_4 group may explain the partial recovery of cellular damage. Higher MDA levels in the CCl_4 +stobadine group than the control group support the histopathologic results of our study. These results also may show stobadine is an antioxidant molecule, but one that has an inadequate protective effect on CCl_4 -based radicals.

Our study is a pioneering step in research regarding the effect of liver damage caused by CCl_4 on the purine degradation pathway. In preventing liver damage caused by CCl_4 , inhibiting key enzymes of this pathway (in particular ADA and XO) may be clinically significant.

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