Biochemically and histopathologically comparative review of thiamine’s and thiamine pyrophosphate’s oxidative stress effects generated with methotrexate in rat liver

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Summary

Background: Oxidative liver injury occurring with methotrexate restricts its use in the desired dose. Therefore, whether or not thiamine and thiamine pyrophosphate, whose antioxidant activity is known, have protective effects on oxidative liver injury generated with methotrexate was comparatively researched in rats using biochemical and histopathological approaches.

Material/Methods: Thiamine pyrophosphate+methotrexate, thiamine+methotrexate, and methotrexate were injected intraperitoneally in rats for 7 days. After this period, all animals’ livers were excised, killing them with high-dose anesthesia, and histopathologic and biochemical investigations were made.

Result: Biochemical results demonstrated a significant elevation in level of oxidant parameters such as MDA and MPO, and a reduction in antioxidant parameters such as GSH and SOD in the liver tissue of the methotrexate group. Also, the quantity of 8-OHdG/dG, a DNA injury product, was higher in the methotrexate group with high oxidant levels and low antioxidant levels, and the quantity of 8-OHdG/dG was in the thiamine pyrophosphate group with low oxidant levels and high antioxidant levels. In the thiamine and control groups, the 8-OHdG/dG rate was 1.48±0.35 pmol/L (P>0.05) and 0.55±0.1 pmol/L (P<0.0001). Thiamine pyrophosphate significantly decreased blood AST, ALT and LDH, but methotrexate and thiamine did not decrease the blood levels of AST, ALT and LDH. Histopathologically, although centrilobular necrosis, apoptotic bodies and inflammation were monitored in the methotrexate group, the findings in the thiamine pyrophosphate group were almost the same as in the control group.

Conclusions: Thiamine pyrophosphate was found to be effective in methotrexate hepatotoxicity, but thiamine was ineffective.

key words: thiamine pyrophosphate • thiamine • methotrexate • hepatotoxicity • rat
**BACKGROUND**

Methotrexate, a folic acid antimetabolite, is used in treatment of many types of cancer as a chemotherapeutic agent; methotrexate also is used in treatment of chronic inflammatory diseases such as dermatomyositis, sarcoidosis, psoriasis and rheumatoid arthritis [1]. However, administration of high-dose methotrexate (in leukemia) or long-term use may cause progressive fibrosis and liver injury progressing to cirrhosis [2]. Clinically, hepatotoxicity occurring in long-term use of methotrexate still remains an important factor restricting its use in desired doses [3].

Therapeutic and hepatotoxic effects of methotrexate are attributed to the inhibition of conversion of folic acid to tetrahydrofolate [4], but there are also studies concluding that methotrexate toxicity arises from different mechanisms [1,5]. Some studies showed the importance of oxidative stress in the mechanism of methotrexate toxicity on liver and other organs [6,7]. Others have found the suppression of enzymatic and non-enzymatic antioxidant levels such as glutathione (GSH), superoxide dismutase (SOD), and the elevation of enzymatic and non-enzymatic oxidant levels such as myeloperoxidase (MPO) and malondialdehyde (MDA), in hepatic, renal and intestinal tissues of experimental animals given methotrexate [8]. Furthermore, residual oxygen radicals were reported to cause oxidative injury of DNA by reacting with DNA [9,10]. These findings suggest that antioxidant treatment may be helpful in prevention of methotrexate toxicity. Therefore, several studies have investigated reducing hepatotoxicity with antioxidants without adding folic acid to methotrexate therapy [8,11]. Because methotrexate toxicity arises from various mechanisms, studies on untested drugs are still continuing in attempts to prevent toxicity. Thiamine pyrophosphate, which we have tried in our study for hepatotoxicity of methotrexate, is an active metabolite of thiamine. Thiamine pyrophosphate is formed by phosphatization of thiamine with thiamine pyrophosphokinase in the liver. Thiamine increases antioxidant formation and NADPH levels, using the pentose phosphate pathway [12]. The antioxidant property of thiamine pyrophosphate is unknown. The biological activity and indications of thiamine pyrophosphate are reported to be different from that of thiamine [13,14].

In the literature, we found no information regarding to the protective effects of thiamine and thiamine pyrophosphate on methotrexate-induced hepatic toxicity. Therefore, the purpose of our study was determine, biochemically and histopathologically, whether thiamine and thiamine pyrophosphate (currently untried) in methotrexate hepatotoxicity has protective effects on methotrexate-induced hepatotoxicity in rats.

**Material and Methods**

**Animals**

Rats used in this study were obtained from the Medical Experimental Application and Research Center, Ataturk University. A total of 28 albino Wistar-strain male rats with a weight varying between 223–240 g were used in the experiment. Animals were housed at the room temperature (22°C) in groups before the experiment. Animals were housed at the room temperature (22°C) in groups before the experiment.

**Chemical agents**

Thiopental sodium was provided by IE Ulagay-Turkey and methotrexate was obtained from Med-drug-Turkey. Thiamine and thiamine pyrophosphate were obtained from Biopharma-Russia.

**Experimental procedure**

Rats in one group (n-7) received 25 mg/kg of thiamine injected intraperitoneally (IP), and rats in the other group (n-7) received 25 mg/kg dose of thiamine pyrophosphate, also injected IP. Distilled water as a solvent was injected in the same way for the methotrexate and control groups (n-7). One hour after administration of drugs, a 5 mg/kg dose of methotrexate was injected IP for all rats groups except the control group. Distilled water as a solvent (instead of methotrexate) was injected IP into control group rats. This procedure was repeated daily for 7 days. After this period, animals were anesthetized with sodium thiopental IP (25 mg/kg). All animals were killed, their livers were excised, and histopathologic and biochemical investigations were made. Histopathologic and biochemical results obtained from thiamine, thiamine pyrophosphate and methotrexate groups were evaluated and compared with the control group.

**Biochemical analysis**

**Total GSH determination**

The amount of GSH in the tissue was measured according to the method of Sedlak and Lindsay [15]. The tissue surface of the liver was collected, weighed, and then homogenized in 2 mL 50 mM Tris-HCl buffer containing 20 mM EDTA and 0.2 mM sucrose, pH 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid, and the precipitate was removed by centrifugation at 4200 rpm for 40 min at 4°C. The supernatants were used to determine GSH using 5,5'-dithiobis (2-nitrobenzoic acid). Absorbance was measured at 412 nm using a spectrophotometer.

**Determination of MPO activity**

MPO activity was measured according to the modified method of Bradley et al. [16]. The homogenized samples were frozen and centrifuged at 1500 g for 10 min at 4°C. MPO activity in the supernatants were determined by adding 100 µL of the supernatant to 1.9 mL of 10 mmol/L-phosphate buffer (pH 6.0) and 1 mL of 1.5 mmol/L o-dianisidine hydrochloride containing 0.0005% (wt/vol) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a UV-vis spectrophotometer.

**Determination of lipid peroxidation or MDA formation**

The concentrations of tissue lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test [17]. Briefly, the rat livers were promptly excised and rinsed with cold saline. To minimize the possibility of interference of hemoglobin with free radicals, any blood adhering to the tissue was carefully removed. The tissue was weighed, and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added to a solution containing 0.2 mL of 80 g/L...
sodium lauryl sulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-hydroxybutyrate, and 0.3 mL distilled water. The mixture was incubated at 98°C for 1 hr. Upon cooling, 5 mL of n-butanol: pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatants was measured at 552 nm.

### Superoxide Dismutase (SOD) analysis

Measurements were performed according to Sun et al. [18]. When xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple colored-formazan dye occurs. The samples were weighed and homogenized in 2 mL of 20 mM phosphate buffer containing 10 mM EDTA at pH 7.8. The samples were centrifuged at 6000 rpm for 10 min and then the supernatants were used as assay samples. The measurement mixture, containing 2500 µL measurement mixture (0.3 mM xanthine, 0.6 mM EDTA, 150 µM NBT, 0.4 M Na2CO3, 1 g/L bovine serum albumin), 500 µL supernatant and 50 µL xanthine oxidase (167 U/L) was vortexed, and then it was incubated for 10 min. At the end of the reaction, formazan is produced. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme is present, there is less least O2·− radical that reacts with NBT.

### ALT (alanine amino transferase)

Venous blood samples were collected into tubes without anticoagulant. Serum was separated by centrifugation after clotting and stored at −80°C until assayed. ALT, AST and LDH (P→L) activity levels were determined in the Cobas 8000 (Roche) photometrical system with colorimetric method.

Pyridoxal-5’-phosphate method, according to the International Federation of Clinical Chemistry (IFCC), catalyzes the reaction between 3,4 ALT L-alanine and 2-oxoglutarate. Lactate dehydrogenase (LDH) of pyruvate L-lactate and NAD⁺ generated by the catalyzed reaction is reduced to NADH. Pyridoxal phosphate acts as a coenzyme in the amino transfer reaction and ensures full activation of the enzyme.

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\text{ALT catalytic activity is directly proportional to the rate of oxidation of NADH.}
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### AST (aspartate aminotransferase)

The pyridoxal-5’-phosphate method, according to the International Federation of Clinical Chemistry (IFCC), is as follows: In the sample of 3,4 AST oxaloacetate and L-glutamate for the formation of L-Aspartate, an amino group with a 2-oxoglutarate catalyzes the transfer. Then oxaloacetate malate dehydrogenase (MDH) will react with the presence of NAD⁺. Pyridoxal phosphate acts as a coenzyme in the amino transfer reaction.

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\text{NADH oxidation rate is proportional to the catalytic activity of AST.}
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Histopathologic analysis

Livers excised from rats were fixed in 10% neutral buffered formalin solution. Sections 5 micrometers in thickness were obtained in paraffin blocks prepared after routine tissue follow-up. All sections obtained were stained with Hematoxylin and Eosin stain, and were evaluated under a light microscope (Olympus CX 51). Histopathologically, liver lobules and the portal area were investigated. Representative microphotographs demonstrated the liver histopathology belonging to experimental animals.

Statistical analysis

All data were analyzed by one-way ANOVA using SPSS 18.0 software. Differences among groups were obtained using the Tukey multiple comparison test and significance was declared at p<0.05.

RESULTS

Results of MDA, MPO, GSH, SOD measurement

GSH, SOD, MDA and MPO levels in the liver tissue of methotrexate group was measured as 5.80±0.06 nmol/g protein, 135.71±1.15 nkat/g protein, 6.44±0.11 µmol/g protein, 55.80±1.80 nkat/g protein, respectively. In the liver tissue of rats receiving thiamine+methotrexate, they were 1.90±0.14 nmol/g protein, 64.06±3.20 nkat/g protein, 5.85±0.13 µmol/g protein and 45.54±3.11 nkat/g protein, respectively. In the control group, GSH, SOD, MDA and MPO values were 4.48±0.09 nmol/g protein, 114.51±4.39 nkat/g protein, 2.37±0.09 µmol/g protein, 14.35±0.30 nkat/g protein, respectively.

Results of AST, ALT, LDH measurement

Figures 1–3 show that while blood AST, ALT and LDH quantities of methotrexate group rats were 3478.57±90.69, 1142.86±33.83 and 32859.38±2759.78 µmol/g protein, and 13.82±0.77 nkat/g protein, respectively. In the liver tissue of rats receiving thiamine+methotrexate, they were 1.52±0.06 nmol/g protein, 52.90±1.85 nkat/g protein, 6.44±0.11 µmol/g protein and 55.80±1.80 nkat/g protein, respectively. In the control group, AST, ALT and LDH values were 4.48±0.09 nmol/g protein, 114.51±4.39 nkat/g protein, 2.37±0.09 µmol/g protein, 14.35±0.30 nkat/g protein, respectively.

Table 1. The MDA and GSH level, MPO and SOD activity in Thiamine pyrophosphate + methotrexate, thiamine + methotrexate, methotrexate and control groups. Results are the means ± Standart error of the mean.

| Groups | GSH nmol/g protein | SOD nkat/g protein | MDA nmol/g protein | MPO nkat/g protein |
|--------|--------------------|--------------------|--------------------|--------------------|
| TPP + methotrexate | 5.80±0.07 | 135.71±1.15 | 2.12±0.21 | 13.82±0.77 |
| p | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Thiamine + methotrexate | 1.90±0.14 | 64.06±3.20 | 5.85±0.13 | 45.54±3.11 |
| p | <0.05 | <0.001 | <0.001 | <0.001 |
| Methotrexate | 1.52±0.06 | 52.90±1.85 | 6.44±0.11 | 55.80±1.80 |
| p | – | – | – | – |
| Control | 4.48±0.09 | 114.51±4.39 | 2.37±0.09 | 14.35±0.30 |
| p | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

Figure 1. The AST activities in methotrexate, control, TPP (thiamine pyrophosphate) + methotrexate and thiamine + methotrexate rat groups. Results are the means ± Standart error of the mean.

Figure 2. The ALT activities in methotrexate, control, TPP (thiamine pyrophosphate) + methotrexate and thiamine + methotrexate rat groups. Results are the means ± Standart error of the mean.

Figure 3. The LDH activities in methotrexate, control, TPP (thiamine pyrophosphate) + methotrexate and thiamine + methotrexate rat groups. Results are the means ± Standart error of the mean.
They were 2152.38±31.80 (P<0.0001), 310.27±22.48 (P<0.0001), and 15053.57±944.77 nkat/L, (P<0.0001) in the group of thiamine pyrophosphate + methotrexate, and 3385.71±110.70 (P>0.05), 959.82±77.97 (P<0.05) and 30064.73±834.06 nkat/L, (P>0.05) in the thiamine+methotrexate group. In the control group, AST, ALT and LDH were 2076.19±49.98 (P<0.0001), 272.32±14.81 (P<0.0001) and 14866.07±1534.89 nkat/L (P<0.0001), respectively.

Results of 8-OHdG/dG Measurement

While 8-OHdG/dG quantity in the methotrexate group was 1.6±0.29 pmol/L, this quantity was 0.58±0.05 (P<0.01), 1.48±0.25 (P>0.05), 0.55±0.04 (P<0.01) pmol/L in the thiamine pyrophosphate, thiamine and control groups, respectively.

Pathologic results

Hepatic tissue of the control rat group

Figure 4 shows normal hepatic histology in the control group.

Rat hepatic tissue in the methotrexate group

As can be seen from Figure 5A, focal necrosis (star) dilatation in the central vein (arrow), accompanied by inflammatory cells, were monitored in the methotrexate group. Furthermore, many apoptotic bodies with condensed cytoplasm and with peripheralized and pyknotic nuclei were seen (Figure 5B).

Hepatic tissue in rats given thiamine+methotrexate

In the group given thiamine, inflammation (Figure 6A), apoptotic bodies (Figure 6B, arrow) and focal necrosis (Figure 6B, star) were monitored in the interstitial area. No dilatation was encountered in the central vein.

Hepatic tissue from rats given thiamine pyrophosphate + methotrexate

In the group given thiamine pyrophosphate, a histopathological appearance close to normal was present, and we found no apoptotic bodies, necrosis, inflammation or dilatation in the central vein (Figure 7A, B).

Discussion

We studied the effect of thiamine and thiamine pyrophosphate on oxidative stress generated with methotrexate, comparing biochemically and histopathologically findings. In physiological conditions, free oxygen radicals forming in cells and the antioxidant defence system are in equilibrium. Disturbance of this equilibrium in favor of oxidants causes oxidative stress. In the hepatic tissue of the methotrexate group, levels of antioxidants such as GSH and SOD were lower than in the control group; MDA, MPO, serum AST, ALT and LDH were higher. These trial results demonstrate that the oxidant/antioxidant equilibrium is impaired in favor of oxidants, and causes hepatic oxidative stress in the hepatic tissue of the methotrexate group. Jahovic et al showed that methotrexate decreases GSH levels in the hepatic and other tissues of rats and increases MPO activity, indicating inflammatory response, and levels of MDA indicating lipid peroxidation [8]. Methotrexate was reported
to decrease GSH quantity by inhibition of nicotinamide adenine diphosphate (NADP). NADP is used to produce GSH by glutathione reductase enzyme [23]. Reduced GSH depends on NADP inhibition and cannot protect hepatocytes against reactive oxygen radicals, and this causes hepatocyte injury [7]. SOD is another endogenous factor protecting tissues against oxidative injury. SOD is the only protective enzymatic mechanism against toxic effects occurring with superoxide radicals. SOD was reported to reduce mediators of oxidative stress and inflammatory response [24, 25].

Jahovic et al. found an elevation in MDA and MPO levels in injured hepatic and other tissues given methotrexate [26]. MDA is a product of lipid peroxidation and causes cross-binding of components in the membrane and cell injury by effecting ion exchange in cell membranes [27]. In the experimental study by Celik et al., MDA quantity was reported to increase in parallel with increased injury [28]. Increasing free radicals in oxidative stress causes neutrophil activation; activated neutrophiles over-secrete MPO in the region of injured tissue generated by free radicals. This event causes even more exacerbation of tissue injury [29]. As seen from our study, methotrexate increases levels of MPO, which is an indicator of neutrophil infiltration. If free radicals occur in a region very close to DNA molecules, DNA molecules may be easily damaged by oxidant radicals [30]. Free radicals enable occurrence of 8-hydroxyguanin (8-OHGua), which is a DNA injury product, by reacting with DNA [10]. As 8-OHGua was accepted as an important indicator reflecting oxidation of DNA [31], 8-OHGua quantity in the animal liver of the methotrexate group significantly increased in proportion to the control group; this event may have arisen from DNA oxidative stress in the hepatic tissue given methotrexate.

In the methotrexate group, hepatic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were significantly higher than in the control group. With previous studies, methotrexate was also shown to elevate hepatic enzymes [7]. Hepatic enzymes such as AST, ALT and LDH are the tests associated with hepatocellular injury. ALT and AST are important tests for determining hepatic injury [32]. In hepatic injury, LDH, AST, ALT and significant elevation in oxidant parameters were demonstrated to be prevented with antioxidants [33]. In our study, biochemical trial results obtained from the methotrexate group were also supported by histopathological findings. LDH, AST, ALT activity, oxidant...
parameters and histopathological injuries such as inflammation, dilatation in the central vein, apoptotic bodies and focal necrosis in the methotrexate group where DNA injury product is high were more explicit. While thiamine pyrophosphate prevented biochemical and histopathological disorders occurring with methotrexate, they were not prevented with thiamine. Inflammation, dilatation in the central vein, apoptotic bodies and focal necrosis were seen in the thiamine group and the methotrexate group. Methotrexate was also shown to exacerbate apoptosis in previous studies [34]. Apoptosis and necrosis may active with the same stimulant: it was shown that if the intracellular ATP quantity is adequate, apoptosis develops; if not adequate, necrosis develops [35]. Kaufman et al found that DNA destruction is a biochemical sign of apoptosis [36]. Results in the literature agree with our biochemical and histopathological trial results. We stated in the introduction that thiamine has an antioxidant property, and we found no data from the literature associated with antioxidant activity of thiamine pyrophosphate. Lukienko et al proved that thiamine has an antioxidant property. [37]. But, in our study, the antioxidant property of thiamine was not determined in oxidative stress generated with methotrexate.

**Conclusions**

As a result, thiamine pyrophosphate was found to be efficient in methotrexate hepatotoxicity, but thiamine was inefficient. The inefficiency of thiamine may have arisen from its inability to prevent the elevation of MDA and MPO levels with methotrexate and the reduction of GSH and SOD levels.

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