

Biochemical Investigation of the Protective Effect of Thiamine Pyrophosphate on Methotrexate-Induced Gastrotoxicity in Rats

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ABSTRACT

Aim: The aim of the study is to investigate the protective effect of thiamine pyrophosphate on methotrexate-induced gastrotoxicity biochemically in rats and to compare it with thiamine. **Methods:** Rats were divided into four groups as sham (SG), thiamine+ methotrexate (TAMTX), thiamine pyrophosphate+ methotrexate (TPMTX) and Methotrexate (MTX) groups. 5 mg/kg methotrexate was administered to 24 hr-fasted rat groups by oral gastric tube except sham rats. 20 mg/kg thiamine was injected to TAMTX group and 20 mg/kg thiamine pyrophosphate was injected to TPMTX group i.p. 5 min before the methotrexate administration. Equal amount distilled water was injected i.p. to SG and MTX groups. 6 hr after the methotrexate administration, the rats were sacrificed with high dose thiopental anesthesia (50 mg/kg). The stomachs of the rats were extracted and MDA, MPO, GSH and SOD analyzes were performed. **Results:** Biochemical results were evaluated by comparing among groups. While tGSH and MDA levels and MPO and SOD activities were 4.2 ± 0.6 , 10.0 ± 0.9 , 7.4 ± 0.8 , 5.2 ± 0.9 $\mu\text{mol/g}$ in MTX group, these parameters were 11.0 ± 1.0 , 3.4 ± 0.4 , 3.3 ± 0.5 , 13.2 ± 1.1 $\mu\text{mol/g}$ in SG group in the gastric tissue. These parameters were measured as 4.8 ± 0.8 , 8.7 ± 0.8 , 5.9 ± 0.5 , 6.1 ± 0.8 $\mu\text{mol/g}$ in TAMTX group and 10.8 ± 0.8 , 4.0 ± 0.5 , 3.6 ± 0.4 , 10.5 ± 0.9 $\mu\text{mol/g}$ in TPMTX group. **Conclusion:** The results showed that thiamine pyrophosphate prevented methotrexate induced oxidative damage in the gastric tissue, but thiamine could not prevent.

Key words: Gastrotoxicity, Methotrexate, Thiamine, Thiamin pyrophosphate, Ulcer.

INTRODUCTION

Methotrexate is an analogue of folic acid and it is used commonly either as a chemotherapeutic agent at various cancer types or at some inflammatory diseases such as psoriasis, dermatomyositis, sarcoidosis and rheumatoid arthritis.¹ But serious side effects can be seen by the use of methotrexate.^{2,3} It was reported that the tissues which have rapidly divided cells such as gastrointestinal tract were significantly affected from methotrexate.⁴ Mucosal cell structure changes caused by methotrexate make gastrointestinal side effects like vomiting and diarrhea. For that reason, side effects especially gastric toxicity seen during the methotrexate

treatment is the most important cause of the refuse of the therapy. Jahovic *et al.* showed that there has been a decrease in Glutathione levels (GSH) known as endogenous antioxidant and a significant increase in the levels of Malondialdehyde (MDA) and Myeloperoxidase (MPO) activity that is the indicator of inflammatory response in blood, liver, kidney, small bowel and stomach tissue in a single dose of 20 mg/kg methotrexate administered rats.⁵ Literature information suggests that antioxidant therapy may be useful in preventing and reducing the toxic effects of methotrexate on the stomach. In this study we tried to

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prevent the gastric toxicity of methotrexate by using thiamine pyrophosphate which is the active metabolite of thiamine. Thiamine pyrophosphate is formed by phosphorylation of thiamine in the liver with pyrophosphokinase.⁶ Thiamine, although known to have antioxidant properties,⁷ couldn't prevent the oxidative damage generated by the ischemia-reperfusion injury in rat ovaries, however thiamine pyrophosphate was found to prevent it.⁸

At a scan of literature any research for the prevention of gastric damage due to methotrexate by thiamine pyrophosphate couldn't be found. Therefore, the aim of our study is to investigate the effect of thiamine pyrophosphate on the oxidative damage made by methotrexate in rat stomach biochemically and compare it with thiamine.

Experimental

Experimental Animals

In this study, a total of 24 Albino Wistar male rats weights ranging from 200-210 g were used. Animals were obtained from Ataturk University Medical Experimental Research and Application Center. Prior to the experiment, the animals were kept and fed at normal room temperature (22°C) for one week (7 days) in the Department of Pharmacology, Laboratory of Ataturk University Medical Faculty in groups. Our studies being concordant to ethical rules of all stages was approved by the document of Ataturk University Local Ethics Committee for Animal Experiments (AUHA-DYEK) No:B.30.2.AT2A.0.23.85-86 dated 5.08.2011 and by the document of Ataturk University, Management of the Institute of Health Sciences Ethics Committee B.30.2.AT. 0.A1.00 / 00/1968 dated 30.6.2011.

Thiamine pyrophosphate and thiamine's effects on gastric damage created by methotrexate

In this study, a total of 24 albino Wistar rats were used including 6 in each group. Rats were divided into 4 groups, including Methotrexate alone (MTX), thiamine pyrophosphate + methotrexate (MTX TB), thiamine + methotrexate (TAMTX) and sham (SG) groups. Rats were fasted for 24 hr, thiamine pyrophosphate 20 mg / kg to TPMTX group and thiamine 20 mg / kg to TAMTX group were injected intraperitoneally. Equal volume of distilled water was applied in the same way to MTX group and SG. Five minutes later, methotrexate 5 mg / kg was given orally by gavage to all groups (except SG). Six hours after administration of methotrexate, the rats were sacrificed by high doses of anesthesia (sodium thiopental 50 mg / kg). After removing the stomach and evaluating the gastric surface macroscopically of

all animals, all stomachs were sent to the Department of Biochemistry Laboratory, at Faculty of Medicine for the measurement of MDA and tGSH quantities and MPO and SOD activity. Biochemical test results were evaluated by comparing groups.

Biochemical analysis of the stomach tissue

To measure enzyme activity in stomach tissue, stomach tissue homogenates were prepared. In the supernatant obtained from the homogenate tGSH, MDA quantities and SOD and MPO enzyme activities were determined by suitable methods based on the literature.

Preparation of samples

At this stage of the study, 0.2 g from each stomach were excised. Stomachs were homogenized in icy media by completing 2 mL respectively with pH=6 potassium phosphate buffer which contains 0.5% HDTMAB (0.5% hexadecyl trimethyl ammonium bromide) for MPO assay and with 1.15% potassium chloride solution for the determination of MDA and for other measurements pH = 7.5 phosphate buffer. They were then centrifuged at 10,000 rpm for 15 min at +4°C. The supernatant fraction was used as the analysis sample.

Malondialdehyde (MDA) Determination

It is based on measuring spectrophotometrically the absorbance of the pink complex formed by Thiobarbituric Acid (TBA) and MDA at high temperature (95°C) at a wavelength of 532 nm.⁹

Determination of Myeloperoxidase (MPO) Activity

In the determining the activity of MPO enzyme, MPO mediated oxidation reaction with H₂O₂ which includes solution of 4-amino anti pyrene / phenol as a substrate was used.¹⁰

Total glutathione (tGSH) Determination

DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] in the medium is a disulfide chromogen and easily can be reduced by compounds which have sulfhydryl groups. The resultant yellow color is measured spectrophotometrically at 412 nm.¹¹

The determination of superoxide dismutase (SOD) activity

It is based on to measure of the purple coloured compound formazan absorbance at 560 nm which is formed by the reduction of nitro blue tetrazolium by means of O₂• radicals produced by xanthine oxidase added to the reaction medium. The intensity of the reduction reactions depends on the Cu / Zn SOD enzyme activity in

the sample. The much enzyme in the environment, the less radical $O_2\cdot$ which reacts with NBT. Thus, the severity of the purple color of formazan gets much less.¹²

Statistical Analysis

The results obtained from experiments were expressed as average value \pm standard deviation “(x \pm SEM). Significance degree of the differences between groups was determined using one- way ANOVA test. And then Fisher’s post-hoc LSD (least significant differences) was made. All statistical procedures were held in” SPSS for Windows, 18.0” statistics program and *p* value <0.05 was considered significant.

RESULTS

Results of MDA analysis

Methotrexate was significantly increased the MDA concentration in gastric tissue at different rates more than thiamine pyrophosphate, thiamine and sham groups. Thiamine pyrophosphate has decreased the increased MDA by methotrexate when compared to thiamine. The difference between the amount of MDA in the gastric tissue of thiamine pyrophosphate and the sham group was not statistically significant (Table 1, Figure 1).

Results of MPO Analysis

At the MPO activity in the gastric tissue by administering methotrexate to the stomach, a significant increase was recorded. While thiamine pyrophosphate reduces the methotrexate caused MPO activity, thiamine couldn’t reduce. MPO activity difference between thiamine pyrophosphate group and the sham group were meaningless, but MPO activity difference between thiamine and the sham group was significantly important (Table 1, Figure 1).

Results of tGSH analysis

As seen from Table 2, thiamine pyrophosphate prevents GSH decrease made by methotrexate in rat gastric

Table 1: The effects of thiamine and thiamine pyrophosphate on the amount of MDA and MPO in the methotrexate administered gastric tissue of the rats.

Groups (N=6)	MDA (μ mol/gr protein)	P	MPO (u/gr protein)	P
MTX	10.0 \pm 0.9	-	7.4 \pm 0.8	-
TPMTX	4.0 \pm 0.5	<i>p</i> <0.0001	3.6 \pm 0.4	<i>p</i> <0.0001
TAMTX	8.7 \pm 0.8	<i>p</i> >0.05	5.9 \pm 0.5	<i>p</i> >0.05
SG	3.4 \pm 0.4	<i>p</i> <0.0001	3.3 \pm 0.5	<i>p</i> <0.0001

* Results are expressed as mean \pm standard deviation. *p* <0.05 was considered significant.

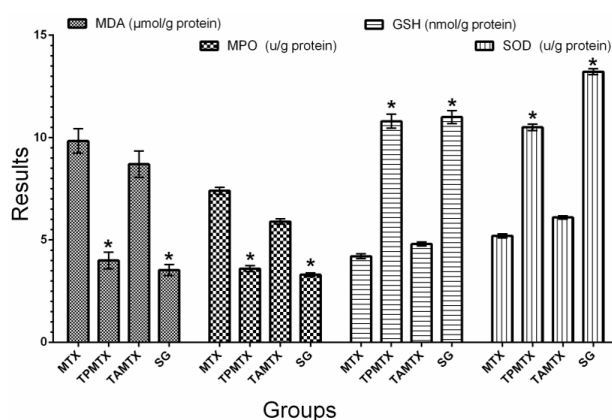


Figure 1: MDA, MPO, tGSH and SOD activity levels in rat gastric tissue in the groups MTX, TAMTX, TPMTX and SG.

Results are expressed as mean \pm standard deviation and were evaluated according to the SG group. (*n*=6), * represents the *p* <0.0001 value.

tissue and thiamine couldn’t prevent. The amount of tGSH in thiamine pyrophosphate group was almost the same as the sham group, the amount of tGSH in thiamine was similar to that in the methotrexate group (Table 2, Figure 1).

Results of SOD analysis

The SOD activity in the group of rats who received thiamine pyrophosphate was found to be significantly higher compared with the group receiving methotrexate and thiamine. It was seen that thiamine was unable to prevent a decrease of SOD by methotrexate (Table 2, Figure 1).

DISCUSSION

In this study, the effects of thiamine and thiamine pyrophosphate in the methotrexate created gastrotoxicity in rats were investigated biochemically. Test results showed that methotrexate causes significant oxidative stress in rat gastric tissue. Today, oxygen free radicals (SOR) in the development of many diseases as well as in the etiopathogenesis of methotrexate gastrotoxicity are held responsible.^{13,14} Antioxidant defense mechanisms in the body and SOR produced by a wide variety of events in physiological conditions are in a balance. By the deterioration of the balance against SOR, oxidative stress occurs causing tissue damage.¹⁵ Oxidative stress begins with the formation of membrane lipid radicals in the cell membrane and then this radicals converts to firstly to lipid hydroperoxides and finally the aldehyde, alkane and toxic products such as MDA.¹⁶ This may lead to changes such as ion transport disorders, changes in enzyme activity and the aggregation of cellular components.¹⁷ In other words, we meet MDA as an oxidant in the continuation and more importantly in

Table 2: The effects of thiamine and thiamine pyrophosphate on the amount of tGSH and SOD in the methotrexate administered gastric tissue of the rats.

Groups (N=6)	tGSH (nmol/gr protein)	P	SOD (u/gr protein)	P
MTX	4.2±0.6	-	5.2±0.9	-
TPMTX	10.8±0.8	<i>p</i> <0.0001	10.5±0.9	<i>p</i> <0.0001
TAMTX	4.8±0.8	<i>p</i> >0.05	6.1±0.8	<i>p</i> >0.05
SG	11.0±1.0	<i>p</i> <0.0001	13.2±1.1	<i>p</i> <0.0001

* Results are expressed as mean ± standard deviation. *p* <0.05 was considered significant.

the exacerbating of oxidative damage. In the MTX group in our study, MDA levels were significantly increased compared to the sham group. The MDA levels in TAMTX group, compared with the MTX group, were showed no statistically significant difference. However, in TPMTX group, MDA levels were lower when compared with the MTX group and with SG group there was no statistically significant difference. These results indicate that in the gastric tissue of MTX group, oxidant / antioxidant balance is disrupted in favor of the oxidants and so, there is oxidative stress in the stomach. In the literature, studies showed that MDA increase is responsible of methotrexate related gastrotoxicity.¹⁸ Significant increase in MPO activity was observed in the gastric tissue of the methotrexate used group. While the difference between MPA activity in groups TAMTX and MTX was meaningless, the difference between groups TPMTX and MTX was significant statistically. There are many studies showing the increase of MPO activity in the damaged stomach tissue.¹⁹ PNL activation causes the release of O₂⁻, OH⁻, H₂O₂ and MPO which are known as cytotoxic radicals.²⁰ Hypochlorous acid and N-chloramines which occurs as a result of the reaction of these radicals with Cl⁻ and MPO initiate the cytotoxic effect in tissue.²¹ As it is known, to limit SOR overproduction and the damage they brought many antioxidant defense mechanisms are developed in the body. Glutathione is one of the endogenous antioxidants and protects the stomach tissue from SOR damage. GSH prevents tissue damage by providing the amount of SOR in the cells at certain concentrations and low levels.²² Decrease of GSH levels in gastric tissue makes gastric damage, increase of it creates a gastroprotective effect.²³ Endogenous GSH plays an important role in maintaining gastric mucosal integrity. Reducing gastric tGSH levels by giving GSH antagonist dietilmalat to the laboratory animals, causes damage in gastric tissue, vise versa the implementation of reduced glutathione prevented the formation of

gastric damage.²⁴ This demonstrates that GSH is an important endogenous antiulcer factor. While no significant difference was observed in GSH levels in the groups of TAMTX and MTX, there was a significant increase in GSH levels in the groups of TPMTX and MTX. Also in this study, it was observed that SOD activity in gastric tissue of the MTX group was significantly lower when compared to the SG group. SOD activity of the TPMTX group was significantly increased when compared to both the TAMTX and the MTX groups. It is known that SOD activity in the injured tissues decrease. It was experimentally proven that diethylthiocarbonate which is a SOD inhibitor, inhibits the secretion of bicarbonate and this inhibition is removed by SOD.²⁵ Our experimental results and knowledge gained from the literature reveals that SOD has gastroprotective effect and indicates that there is a direct connection between SOD and gastroprotection. In a few previously made clinical and experimental researchs thiamine and thiamine pyrophosphate were compared, in the majority of the studies, any distinction weren't made between these two substances.²⁶ The experimental works recently done reported that, while thiamine pyrophosphate could prevent the oxidative tissue damage in ischemia reperfusion injury in the ovary, thiamine couldn't.⁸ These experimental results are consistent with the literature.

CONCLUSION

As a result; in the methotrexate-induced oxidative gastric damage, thiamine and thiamine pyrophosphate were found to act completely differently from each other. Thiamine pyrophosphate has created significant protective effect against methotrexate gastrotoxicity. Thiamine has remained inadequate in preventing the methotrexate gastrotoxicity. This suggests that to administer thiamine pyrophosphate which is the active form of thiamine, instead of thiamine in the methotrexate gastrotoxicity may be more appropriate.

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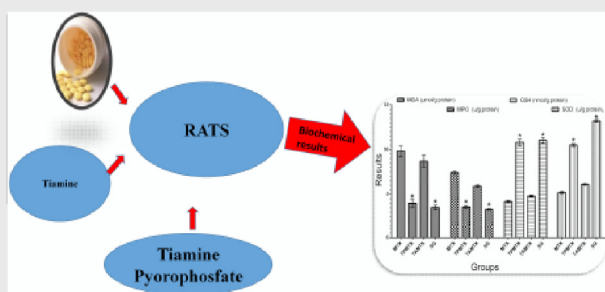
CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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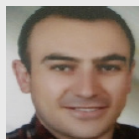
PICTORIAL ABSTRACT



SUMMARY

The current research work shows that the effects of thiamine and thiamine pyrophosphate in the methotrexate created gastrotoxicity in rats were investigated biochemically. Methotrexate causes significant oxidative stress in rat gastric tissue. As a result; in the methotrexate-induced oxidative gastric damage, thiamine and thiamine pyrophosphate were found to act completely differently from each other. Thiamine pyrophosphate has created significant protective effect against methotrexate gastrotoxicity. Thiamine has remained inadequate in preventing the methotrexate gastrotoxicity. This suggests that to administer thiamine pyrophosphate which is the active form of thiamine, instead of thiamine in the methotrexate gastrotoxicity may be more appropriate.

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