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Original Article

The protective effect of intraperitoneal medical ozone preconditioning and treatment on hepatotoxicity induced by methotrexate

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Abstract: The aim of this study is to determine the effects of medical ozone preconditioning and treatment on the methotrexate acute induced hepatotoxicity in rats that has not reports elsewhere. Eighteen rats were randomly assigned into three equal groups; control, Mtx and Mtx with ozone. Hepatotoxicity was performed with a single dose of 20 mg/kg Mtx to group 2 and group 3 at the fifteenth day. The medical ozone preconditioning was administered intraperitonealy in group 3 for fifteen days and more five days after inducing Mtx. The other rats of the group 1 and 2 received saline injection. At the twentyfirst day the blood and the liver tissue samples were obtained to measure the levels of liver enzymes ALT and AST, proinflamatory cytokines TNF-α, IL-1β, malondialdehyde, glutathione and myeloperoxidase. And the histolopatological examination was evaluated for injury score. In our study Mtx administration caused a significant increase on the liver enzymes ALT and AST, the tissue MDA and MPO activity and significant decrease in the tissue GSH. Moreover the both pro-inflammatory cytokines were significantly increased in the Mtx group. Medical ozone preconditioning and treatment reversed all these biochemical parameters and histopathological changes of the hepatotoxicity induced by Mtx. We conclude that medical ozone ameliorates Mtx induced hepatotoxicity in rats.

Keywords: Methotrexate, liver, hepatotoxicity, medical ozone, preconditioning

Introduction

Methotrexate (mtx), analogue of folic acid is an effective cytotoxic chemotherapeutic drug and has been widely used in many malignancies [1, 2] and inflammatory diseases [3-6]. Long-term mtx use or its usage in high doses may cause hepatic steatosis, cholestasis, fibrosis and cirrhosis [7]. According to the hepatotoxicity due to mtx should be the reason for the delay in the treatment.

Medical Ozone is described as three atom molecules of oxygen known as O_3. Ozone is applied in medical therapy using a gas mixture composed of oxygen and ozone. Ozone theraphy has been found useful in the treatment of various diseases including gastroduodenal ulcers, peritonitis, colitis and chronic skin ulcers [8-10]. Repeated ozone administration at nontoxic doses provide an adaptation of tissues to oxidative stress by induction of enzymes or activating the metabolic pathways maintaining an equilibrated redox balance such as increase on glutathione levels and decrease on lipid peroxidation [11]. Furthermore the effect of O_3 on Mtx induced hepatotoxicity has not been studied before. In this present study we aimed to evaluate whether O_3 preconditioning and treatment has any effects on Mtx-induced hepatotoxicity in rats.

Material and methods

Eighteen Wistar albino rats of male gender of 250-300 g were used in this study. The experimental procedures were reviewed and approved by Akdeniz University Local Committee on

Table 1. Ozone effect on biochemical parameters in the serum of control, Mtx, Mtx-Ozone groups. Each group was consisting of 6 rats. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests

	Control	Mtx	Mtx-Ozone
TNF-α (pg/ml)	12.33±3.07	29.50±2.73**	18.16±2.04*,++
IL-1β (pg/ml)	12.50±1.87	29.16±2.63**	17.16±1.16*,++
ALT (IU/L)	1.71±0.14	15.20±0.96**	3.56±0.59*,++
AST (IU/L)	2.55±0.28	18.08±1.61**	4.68±0.63*,++

Data are mean ± SD. *P<0.01, **P<0.001 compared to control group. ++P<0.001 compared to Mtx group.

Animal Research Ethics (2015.02.07). The rats maintained at a constant temperature (22°C) with a 12-h light-dark cycle and randomly divided into three equal groups. Group 1 was the control group receiving only physiological saline. Group 2 was the Methotrexate group receive a single 20 mg/kg dose of Mtx (Onco-Tain; Faulding Pharmaceutics Plc, Leamington Spa, UK) intraperitoneally for induce hepatotoxicity at the end of fifteenth day and receiving 5 mL of saline intraperitoneally everyday for twenty days. Group 3 was the Methotrexate-medical ozone group preconditioned with 5 mL of medical ozone everyday for fifteen days and receiving a single dose of Mtx at the sixteenth day and also treated with 5 mL medical ozone (OM-302, Ozone generator, Sedecal, Spain) intraperitoneally everyday for more five days at dose of 25 mcg/mL. At the end of the twenty-first day the rats were sacrified with decapitation after ether anesthesia and the blood samples and liver tissues were obtained. The levels of tumour necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) from blood and the levels of malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) from liver tissue samples were analysed. Histopathological analysis and the liver tissue injury score was performed by an expert pathologist under a light microscope.

Analysis of TNF-α and IL-1β

TNF-α and IL-1β from plasma were analysed by the enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium).

Analysis of ALT and AST

The liver enzymes ALT and AST plasma concentrations were measured in accordance with the diagnostic methods in samples obtained from all groups of rats serum. Activities were expressed as IU/L.

Measurement of malondialdehyde and glutathione levels

The liver tissue samples were homogenized in ice cold 150 mm KCl. The MDA levels (nmol MDA/g tissue) were assayed for the products of lipid peroxidation [12]. The GSH levels (nmol GSH/g tissue) were measured using the method of Elman [13]. GSH is reacted with 5,5-ithiobis-2-nitrobenzoic acid resulting in the formation of a product which has a maximal absorbance at 410 nm.

Measurement of myeloperoxidase activity

Measurement of the tissue-associated MPO (U MPO/g tissue) activity according to the Hillegas et al. [14] procedure. Liver tissue samples were homogenized in 50 mm potassium phosphate buffer (PB, pH 6.0) and homogenates were centrifuged at 41 400 g for 10 min; pellets were suspended in 50 mm PB containing 0.5% hexadecyltrimethylammonium bromide. After three cycles of freezing and thawing, with the sonication between the cycles, liver samples were centrifuged at 41 400 g for 10 min. Volumes of 0.3 ml were added to 2.3 ml of reaction mixture containing 50 mm PB, o-dianisidine, and 20 mm H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 min.

Histopathological analysis

Liver specimens were fixed in 10% buffered formaldehyde. One horizontal section from each liver was embedded in paraffin, cut at 3 micrometers and stained with hematoxylin-eosin (H&E) and Periodic Acid-Schiff-diastase (D/PAS). Histopathological analysis was performed by an expert pathologist who was blinded to the experiment. For each tissue, semi-quantitative scales used in the liver were as follows; (1) vascular congestion, (2) degeneration and dilatation of the sinusoids, (3) hydropic degeneration (cellular swelling), (4) vacuolar degeneration of hepatocytes, (5) increase in the number of activated Kupffer cells, (6) portal inflammatory cell infiltration.

Table 2. The comparison of MDA, GSH and MPO levels in liver tissue of control, Mtx and Mtx-Ozone groups. Each group was consisting of 6 rats. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests

	Control	Mtx	Mtx-Ozone
MDA (nmol/g tissue)	29.83±3.43	57.83±3.86***	30.83±3.71^{+++}
GSH (nmol/g tissue)	1816.00±113.72	1547.33±82.26*	1965.33±175.37^{+}
MPO (U/g tissue)	23.66±2.16	45.33±2.06***	29.33±2.16 **,$^{+++}$

Data are mean ± SD. *P<0.01, **P<0.001, ***P<0.0001 compared to control group. ^{+}P<0.01, ^{++}P<0.001, ^{+++}P<0.0001 compared to Mtx group.

The whole microscopic area was examined to score each specimen. Tissue injury was scored using a scoring system. Scores were given as 0 = none, 1 = mild, 2 = moderate and 3 = severe. [15].

Statistical analysis

Statistical analysis was performed by GraphPad Prism 3.0 (GraphPad Software, San Diego, USA). The data were expressed as mean ± standard error of the mean (SEM). Group comparisons were performed with the analysis of variance (ANOVA) followed by Tukey's tests. The P<0.05 was considered as statistically significant.

Results

In the Mtx group, proinflammatory cytokine TNF-α levels were found significantly increased 29.50±2.73 pg/mL (P<0.001) when compared to control (12.33±3.07 pg/mL), while this Mtx-induced rise in serum TNF-α level was abolished 18.16±2.04 pg/mL (P<0.01) with preconditioning with O_3. In the Mtx group when compared to control 12.50±1.87 pg/mL (P<0.001) IL-1β was also found as increased 29.16±2.63 pg/ml (P<0.001). After preconditioning with O_3 following Mtx, IL-1β was also decreased to 17.16±1.16 pg/ml (P<0.01) (**Table 1**). The liver enzymes ALT and AST were also found as increased in Mtx group but both were diminished after O_3 preconditioning.

In accordance with these findings the mean level of MDA, which is a major degradation product of lipid peroxidation seen as increased in all tissues (57.83±3.86 nmol/g tissue) after Mtx administration when compared with the control group (29.83±3.43 nmol/g tissue) (P<0.001), while preconditioning with O_3 to the Mtx group came back to control MDA levels 30.83±3.71 nmol/g tissue (P<0.001, **Table 2**; **Figure 1**).

The major cellular antioxidant GSH levels of liver samples in Mtx group were found lower significantly (1547.33±82.26 nmol/g tissue) (P<0.01). On the other hand, preconditioning with O_3 to Mtx group (1965.33±175.37 nmol/g tissue) restored the GSH levels to control levels (1816.00±113.72 nmol/g tissue) in all liver samples (P<0.01, **Table 2**; **Figure 1**).

Indicator of neutrophil infiltration Myeloperoxidase has significantly higher activity in the liver samples of the Mtx group (45.33± 2.06 U/g tissue) when compared to control (23.66±2.16 U/g tissue) (P<0.0001). On the other hand, preconditioning with O_3 in Mtx group significantly decreased all MPO activity level (29.33±2.16 U/g tissue) (P<0.001, **Table 2**; **Figure 1**).

Histopathological evaluation and average degree of injury scores of the groups in medians were shown in **Table 3**. Liver sections from the control group were normal in histological appearance. The liver sections from the Mtx group showed some histopathological changes such as inflammation around portal vein and lymphoplasmacytic inflammation (**Figure 2**). Ozone preconditioning reduced the histopathological damage score in the Mtx+O_3 group in comparison to the control and Mtx groups. In this group, liver sections showed perivenulary sinusoidal dilatation (**Figure 3**).

Discussion

According to literature search, this is the first study investigating the effect of preconditioning and treatment of medical ozone on experimental Mtx-induced hepatotoxicity in rats. Findings from our study revealed that liver injury ameliorated by intraperitoneal medical ozone administration, while with antioxidant effect the ozone prevented the lipid peroxidation and neutrophil infiltration of the rat liver tissues. The decreasing on tissue MDA levels and MPO activities with an increasing on levels of GSH revealed that ozone preconditioning prevented

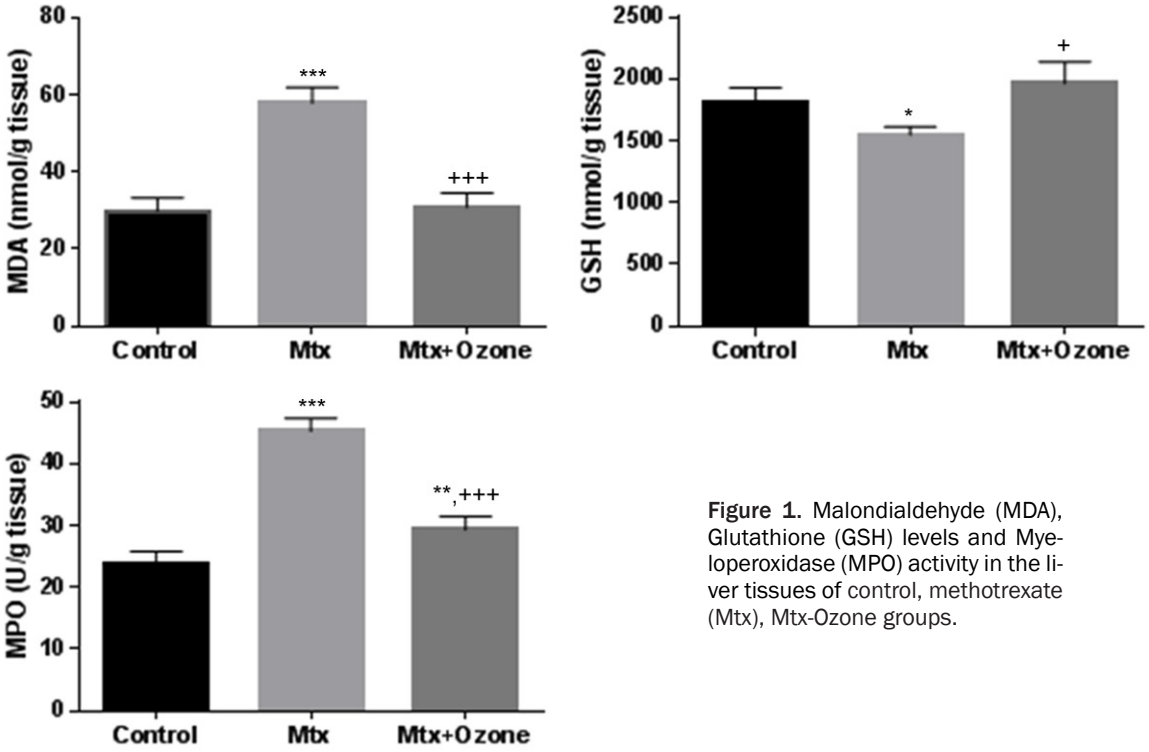

Figure 1. Malondialdehyde (MDA), Glutathione (GSH) levels and Myeloperoxidase (MPO) activity in the liver tissues of control, methotrexate (Mtx), Mtx-Ozone groups.

Table 3. The average degree of histopathologic damage was expressed as medians within each liver section of groups were shown

Parameters	Control	Mtx	Mtx-Ozone
Vascular congestion	0	2	1
Degeneration and dilatation of the sinusoids	0	2	2
Hydropic degeneration (cellular swelling)	0	2	1
Vacuolar degeneration of hepatocytes	0	2	1
Increase in the number of activated Kupffer cells	0	2	1
Portal inflammatory cell infiltration	0	3	2
Total score	0	13	8

liver tissue from oxidative stress. Also ozone preconditioning was significantly improved the changes on liver tissue caused by mtx in rats by decreasing the plasma cytokines and liver enzymes ALT and AST.

Methotrexate is an antimetabolite that competitively inhibite the folic acid metabolism thus impairs the DNA synthesis. 7-hydroxymethotrexate is the major extracellular metabolite of Mttx that is metabolized in the liver by an enzymatic system [7]. With the use of Mtx intracellular amount of polyglutamate increases on the other hand folic acid levels decreased that leads to necrosis of hepatocyte [16]. Hepatotoxic effect of mtx was caused by an increase of its polyglutamate form intracellularly. The hepatotoxic effects of mtx have been reported in many studies [1, 17, 18].

Ozone preconditioning was investigated in many studies on animal models of hepatotoxicity and according to these findings showed ozone as able to induce an adaptation to oxidative stress and promote oxidative preconditioning through the increase and preservation of antioxidant endogenous systems which can be induced by CCl4. It also can produce protection against hepatic and renal ischemia reperfusion injury [19-21]. In a recent study, ozone investigated to provide a protective effect against radiation-induced oxidative damage in the liver [22]. A controlled number and dose of ozone treatments conferred protection against different physiopathological processes mediated by ROS was reported [19].

Ozone preconditioning prevented and thus decreased the serum ALT and AST enzyme activities which are the liver injury indicatives induced by Mtx administration. On the other hand, ozone probably prevented liver necrosis

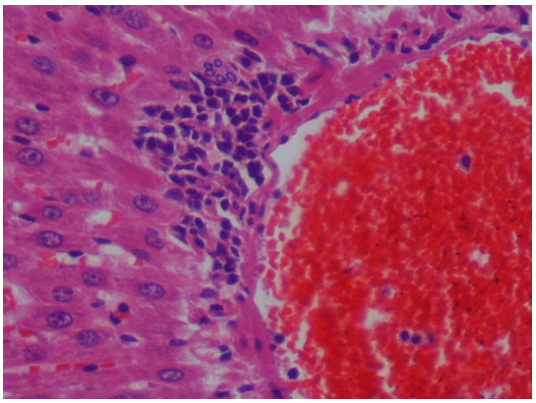

Figure 2. Mtx group; Portal inflammation. Lymphoplasmacytic inflammation and a small biliary ductule is seen in the inset (X10, H/E, inset: X40, H/E).

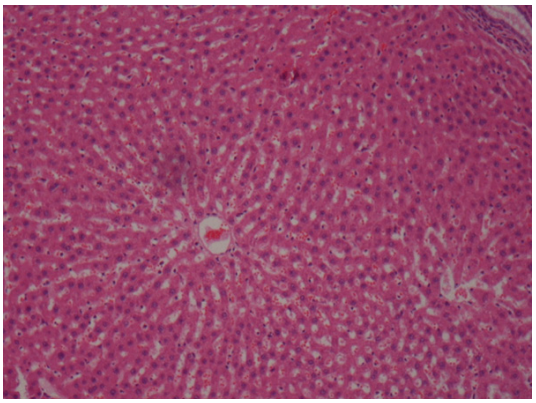

Figure 3. Mtx+O_3 group; In this picture, perivenular (Zone III) area shows sinusoidal dilatation. Liver has the appearance of a nutmeg. Compare sinusoids with the upperright. (X10, H/E).

by modulating the antioxidant defense system, improving oxygen delivery and increasing the release of vascular nitric oxide [23]. In a previous study it was reported that ozone exposure altered the levels of inflammatory cytokines, such as tumor necrosis factor [24], transforming growth factor [25], interferon [26] and interleukin [27]. In our study systemic inflammatory response indicators; plasma TNF-α and IL-1β levels were also found increased due to mtx administration but decreased by ozone preconditioning.

Lipid peroxidation by free oxygen radicals is an important causes of destruction and oxidative damage to cell membranes these containing unsaturated fatty acids, nucleic acids and proteins. It can resulted with methotrexate induced tissue damage [7, 12, 18, 28]. The decreasing on tissue MDA levels revealed that ozone preconditioning prevented liver tissue from oxidative damage.

At previous studies reported that stimulation of antioxidant enzyme activities such as glutathione by ozone prepared the tissue against the reactive oxygen species effects [29, 30]. GSH plays a particularly important role in the maintenance and regulation of the thiol-redox status of the cell [31]. Tissue GSH depletion is one of the primary factors permitting liver tissue damage is associated with oxidative stress caused by mtx in our study. O3 was previously reported to increase glutathione levels in erythrocytes and it may show the same action in hepatocytes, thus increasing the detoxification capacity of these cells [32]. In our study preconditioning with ozone reversed the levels of GSH into normal levels.

It was expected that free oxygen radicals plays an important role in mtx induced liver toxicity [33, 34]. It triggers the leukocytes accumulation in tissue and activates neutrophils to secrete enzymes. And this leads to further tissue damage. Therefore, this MPO an enzyme secreted by neutrophils plays role in oxidant production [35, 36]. In our study MPO activity which is an index of polimorphonuclear leukocyte infiltration and accumulation was found increased. Increased activities of MPO indicate that contributes to Mtx induced oxidative liver injury in rats. Intraperitoneal ozone preconditioning decreased the MPO activity.

In our study as a result preconditioning with intraperitoneal ozone was significantly reduced the liver enzymes ALT and AST, plasma TNF-α and IL-1β levels, MDA levels and MPO activity, increase the GSH levels while histological changes was observed decreased in liver tissue.

Conclusion

In conclusion, preconditioning and treatment with O_3 intraperitoneally ameliorate the liver injury induced by methotrexate in rats.

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Disclosure of conflict of interest

None.

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