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Original Article

The protective effects of alpha lipoic acid on methotrexate induced testis injury in rats

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ABSTRACT

Methotrexate (MTX) is frequently used in the treatment of several diseases including cancers, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, and dermatomyositis. Previously, chemotherapeutic agents have been reported to cause permanent azoospermia and infertility in men. Methotrexate has been also shown to damage the seminiferous tubules of the testicles, lower the sperm count, and cause genetic mutations (in DNA) in sperm. In this study, we aimed to investigate the protective effects of alpha lipoic acid (ALA) on MTX-induced testicle damage in a rat model.

A total of 40 male Wistar Albino rats were used in this study. The rats were divided into four groups including 10 rats in each. The first group (control group) received only saline intraperitoneal (i.p.); the second group (ALA group) was given ALA 100 mg/kg i.p.; the third group (MTX group) received single dose MTX 20 mg/kg i.p.; and the fourth group (MTX + ALA group) received single dose MTX 20 mg/kg i.p. and ALA 100 mg/kg i.p. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), myeloperoxidase (MPO) levels in the testicular tissue and serum testosterone, serum total antioxidant status (TAS) and total oxidant status (TOS) levels were biochemically evaluated. Testicular tissues histopathologically evaluated.

In the MTX group, the MDA, TAS and TOS levels were higher, while the SOD, CAT, GPx, MPO and serum testosterone levels decreased. Compared to the MTX group, the MDA, TAS and TOS levels were lower and the SOD, CAT, GPx, MPO and serum testosterone levels increased in the MTX + ALA group. In the histopathological examination, the mean seminiferous tubule length (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) were found to significantly decrease in the MTX group, compared to the control group. These values were significantly higher in the MTX + ALA group, compared to the MTX group ($p < 0.05$).

In our experimental study, MTX caused severe tissue destruction in testicles by increasing the formation of free oxygen radicals. Based on our study results, we suggest that, as a potent free radical scavenger, ALA can reduce MTX-induced testicular tissue damage thanks to its antioxidant and anti-inflammatory properties.

1. Introduction

Methotrexate (MTX) is a folate antagonist, which is frequently used in several types of cancer and inflammatory processes such as rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus and dermatomyositis [1]. Previously, chemotherapeutic drugs have been reported to cause permanent azoospermia and infertility in men [2]. Methotrexate has been shown to damage the seminiferous tubules of the testicle, lower the sperm count, and cause genetic mutations (DNA damage) in the sperm [3–5].

Atrophy of the testicular seminiferous tubules and apoptosis in spermatocytes have been also associated with increased reactive oxygen species (ROS) [3–5]. In recent studies, antioxidants have been used to reduce the side effects due to MTX administration [4,5]. Oxidative stress has been reported to plays an important role in testicular damage caused by MTX [6].

Alpha lipoic acid (ALA) exists in all types of prokaryotic and eukaryotic cells and plays a major role in the mitochondrial dehydrogenase reaction. In recent years, it has been also paid attention, mainly as an antioxidant and anti-inflammatory agent [7]. Alpha lipoic acid has free radical scavenger properties and direct antioxidant effects on the recycling of other cellular antioxidants. Alpha lipoic acid has

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been also found to be effective in the treatment of various oxidative stress models, such as ischemia-reperfusion, diabetes, cataract formation, neurodegeneration, and radiation injury [8,9].

In the present study, we aimed to investigate the protective effects of ALA on MTX-induced testicle damage in a rat model using histopathological and biochemical parameters.

2. Material and methods

2.1. Experimental protocol

In this experimental study, a total of 40 male Wistar rats (aged between 10 and 12 weeks and weighing between 250 and 300 g) were used. The study was approved by the Ethics Committee of Mustafa Kemal University (MKU) (2016/4-3). All animals were kept in the Experimental Animals Practice and Research Center. Artificial light was maintained for 12-h light and 12-h darkness cycle at 21 ºC, and all animals were fed with standard pellet feed and tap water. All animals were divided into four groups including 10 rats in each:

Group 1 (Control group) each rat received only 0.5 ml volume daily saline intraperitoneal (i.p.) for the following 10 days, Group 2 (ALA group) each rat received only 0.5 ml volume daily ALA 100 mg/kg i.p. for the following 10 days, Group 3 (MTX group) each rat received single dose 0.5 ml volume MTX 20 mg/kg i.p. on the eight day of the experiment [2] and Group 4 (MTX + ALA group) received single dose 0.5 ml volume MTX 20 mg/kg i.p. on the eight day of the experiment and each rat received 0.5 ml volume ALA 100 mg/kg i.p. for the following 10 days. On day 10, all animals were sacrificed by cardiac blood withdrawal method under ketamine/xylazine anesthesia. Testicular tissues and blood samples were stored at −70 °C. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), myeloperoxidase (MPO) levels in the testicular tissue and serum testosterone, serum total antioxidant status (TAS) and total oxidant status (TOS) levels were biochemically evaluated. Testicular tissues were kept in 10% formaldehyde before the histopathological evaluation.

2.2. Chemicals

Methotrexate® 50 mg/5 mL flakon was purchased from Kocak Pharma, Turkey. Alfamine (10%/50 mL) was supplied from Ata Fen, Turkey. Xylazine hydrochloride (100 mg/mL) (Rompun) was purchased from Bayer. Alpha lipoic acid was supplied from Sigma-Aldrich (St Louis, MO, USA, Lot: BCBM3541V). We used (±)-α-Lipoic acid. (±) 1,2-Dithiolane-3-pentanoic acid, 6,8-Dithiooctanoic acid, DL-α-Lipoic acid, DL-6,8-Thioctic acid, Lip (S2) are synonyms. Alpha lipoic acid was dissolved in a physiological saline solution containing 0.5% NaOH, and the pH of the solution was adjusted to 7.4 with HCl.

2.3. Biochemical methods

2.3.1. MDA

The MDA levels were measured by the double-heating method, as described by Draper and Hadley [10]. The principle of this method is the spectrophotometric measurement (at 532 nm) of the final product of lipid peroxidation reaction with thiobarbituric acid. Concentration of MDA was calculated by a calibration curve, which is commercially available (1,1,3,3-tetrametoksipropan, Lot no, MKBP9901V, Sigma-Aldrich) and expressed in μmol/mg protein.

2.3.2. SOD

The SOD activity was measured using the method described by Sun [11]. The principle of the method is the inhibition of nitrobluentetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. The SOD activity was calculated as unit per milligram protein (U/mg protein).

2.3.3. GPx

The GPx activity was measured by the method described by Paglia and Valentine [12]. Enzymatic reaction was initiated with the addition of H_2O_2 into the reaction mixture containing reduced glutathione (GSH), reduced nicotinamide-adenine dinucleotide phosphate ($NADP^+$), and GSH reductase. The GPx activity was quantitated spectrophotometrically by measuring the absorbance changes at 340 nm. The GPx activity was defined as unit (micromoles of oxidized NADPH per min) and expressed in U/mg protein.

2.3.4. CAT

The CAT activity was measured by the method described by Aebi [13]. The decomposition of the H_2O_2 substrate was monitored spectrophotometrically at 240 nm (Schimadzu UV 1601, Japan), and the results were expressed in pg/ml.

2.3.5. Testosterone levels

The MPO and testosterone levels were measured by the enzyme-linked immunosorbent assay (ELISA). The MPO ELISA kit was purchased from Hycult Biotech, and testosterone ELISA kit was purchased from R&D.

2.3.6. TAS, TOS levels

Serum TAS and TOS levels were measured using a novel automated colorimetric measurement method, which was developed by Erel (Rel Assay Diagnostics kits, Mega Tip, Turkey) [14,15]. The TAS levels were expressed in milimolar hydrogen peroxide equivalent per g protein (mmol Trolox Eq/g protein). The TOS levels were expressed in milimolar hydrogen peroxide equivalent per g protein (mmol H_2O_2 Eq/g protein).

2.4. Histopathological methods

Testicular tissues were dissected in the longest dimension and fixed in 10% formaldehyde solution. After fixation for 24 h, the samples with 4-μm thickness were stained with the Hematoxylin & Eosin (H&E) staining. The samples in each group were evaluated under Olympus BX53 microscope (Olympus,Tokyo, Japan) with the method described by Bozlu [16]. In each section, the mean seminiferous tubule length (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) for 20 seminiferous tubules were measured. The MSTD values were measured using an eyepiece micrometer (ZA3262, U-OCMC, 24 mm cross, 10/100×) mounted into one of the eyepiece objectives. At 400× power, the field was 0.44 mm × 0.44 mm, yielding an area of approximately 0.19 mm^2. The MSTD of each testis was expressed in micron. The GECT was calculated by counting and averaging the number of epithelial cells from the basal membrane to the lumen at 90°, 180°, 270°, and 360°. The MBTS was scaled using the Johnsen score. Each tubule was scaled from 0 to 10 according to the epithelial maturation [16].

2.5. Statistical analysis

Statistical analysis was performed using the SPSS version 21.0 (IBM Corp, Armonk, NY, USA). The descriptive statistics were expressed in mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the groups, and the Bonferroni test was used for the *post-hoc* analysis. A p value of < 0.05 was considered statistically significant.