ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (11): 639 - 646
doi: 10.30466/vrf.2025.2045548.4542
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Thiamine attenuates methotrexate-induced reproductive toxicity in adult male mice

Sally Adnan Mousa Al-Rekabi[1], Rasoul Shahrooz[1]*, Shapour Hasanzadeh[1], Gholamreza Najafi[1], Hassan Malekinejad[2], Khalied Yassen Zakair[3]

[1] Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Pharmacology and Toxicology, School of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran; [3] Department of Medical Laboratory Techniques, Kut Technical Institute, Middle Technical University, Baghdad, Iraq.

Article Info

Article history:

Received: 12 November 2024
Accepted: 22 April 2025
Available online: 15 November 2025

Keywords:

Fertility
Methotrexate
Mice
Sperm
Thiamine

Abstract

Chemotherapy agents impose harmful side effects beside their therapeutic properties. This study aimed to uncover the ameliorative effects of thiamine (VitB1) on sperm characteristics and *in vitro* fertilizing ability in methotrexate (MTX) exposed mice. For this purpose, 36 apparently healthy adult male mice were randomly divided into six groups (n = 6), including control (0.10 mL normal saline *per* day; intraperitoneally [IP]), sham (10.00 mg kg^{-1} *per* week MTX; IP), positive control group (100 mg kg^{-1} *per* day VitB1; IP), and three experimental groups (25.00, 50.00, and 100 mg kg^{-1} *per* day; IP VitB1 plus MTX, respectively; IP). Then, sperm parameters and the *in vitro* fertilizing potentials were assessed. The sperm count, motility, and viability in the sham group showed a significant decrease in comparison with the VitB1-treated and control groups. In the VitB1 100 mg kg^{-1} + MTX group, there was a significant increase in sperm count in comparison with the sham group. The sperms with DNA damages and immature sperms in the sham group significantly increased in comparison with the positive control and control groups. *In vitro* fertilization and embryonic development in the sham group were decreased in comparison with both the control and VitB1 groups. In VitB1 50.00 mg kg^{-1} and 100 mg kg^{-1} + MTX groups, there was an increase in the fertilization rate, along with embryonic development promotion. According to the results of this study, thiamine ameliorates toxic effects of MTX on sperm traits and *in vitro* fertilizing potentials by constraining oxidative stress.

Introduction

Since beginning of 20th century, the chemotherapy as a choice of cancer treatment was commenced, and methods of screening of chemicals for their carcinogenesis through experimental methods in rodents were introduced.[1] Thus, improvements in cancer treatment over the past few decades have led to improved survival outcomes in patients with malignant diseases. For men at any age, one of the key concerns after cancer treatment is their ability to procreate.[2] Although different methods of treatments, such as chemotherapy, are effective in the management of different types of cancers, they may cause the death of normal proliferating germ cells, severely impairing fertility in an agent- and dose-dependent ways.[3]

Methotrexate (MTX; 4-amino-10-methylfolic acid) is a folic acid antagonist that has the property of treating cancer cells.[4] This drug competes with folic acid in cancer cells and induces cell death by triggering a cellular folic acid deficiency.[5] The MTX can cause severe side effects in different body tissues. Its toxicity has been reported in various systems, including digestive, circulatory, and central nervous systems.[6,7]

In the reproductive system, especially testes, rapidly dividing cells of germinal epithelium have been reported to be affected by MTX, and for this reason, the side effects in reproductive system, like testicular toxicity, are one of the most significant factors of treatment failure.[8] There are worries about men's fertility and the consequences in their partner's pregnancy caused by the drug's noxiousness. Methotrexate destroys or severely damages dividing cells, a process that occurs continuously during sperm production.[9] The MTX causes testicular toxicity through oxidative stress or direct toxic effects. There have been many case reports of reversible infertility in men who have used MTX.[10] Treatment with MTX leads to a sharp

***Correspondence:**

Rasoul Shahrooz. DVM, DVSc
Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: r.shahrooze@urmia.ac.ir

decrease in the sperm population, an increase in sperm disorders, including malformed sperms and sperms DNA breakages and damages.[11] According to previous reports, subsequent to MTX therapy, an increase occurs in intra-cellular reactive oxygen species (ROS); therefore, oxidative stress is one of the main consequence.[12] The distinctive plasma membrane structure of sperm contains significant levels of polyunsaturated fatty acids, providing the flexibility needed for the oocyte penetration. Free radicals can easily attack this membrane structure.[13] Reduced sperm motility appears to be due to a cascade of lipid peroxidation, compromising the integrity of the cell membrane, subsequently reducing fertility.[14] Fortunately, according to the reports, after ending the use of this drug, compensation occurs; thus, quantity and quality of sperm more likely return to the normal levels.[15]

Thiamine (VitB1) is an essential micro-nutrient for mammals and is found in abundance in some foods. This water-soluble vitamin is commercially manufactured as a food supplement or medicine.[16,17] As a result of cellular phosphorylation, VitB1 is converted into thiamine pyrophosphate and participates in cellular enzymatic processes related to carbohydrate, lipid, and branched amino acid metabolism as a cofactor. In addition to its vital role in energy metabolism, it also has catalytic activity in hexose-monophosphate pathways.[18] Therefore, this substance acts as an auxiliary agent and energy source for many cellular enzymes, and also improves the level of antioxidants by participating in the pentose phosphate cycle that produces nicotinamide adenine dinucleotide phosphate.[18] Despite of its importance in oxidative metabolism and as a metabolic requirement, the role of this vitamin in spermatogenesis and male infertility has not been investigated so far.[18] The aim of this study was to investigate the repro-protective effects of VitB1 on the MTX-induced sperm and fertility disorders in adult male mice.

Materials and Methods

Animal model. For this study, 36 adult male Balb/c mice (weight: 25.00 - 30.00 g and age: 8 - 12 weeks) were selected and randomly divided in to six groups. Initially, mice were acclimated for one week before starting the 36-day treatment protocol and all groups experienced equal standard condition (23.00 ± 3.00 ˚C, 12-hr light/dark cycle, 30.00 to 60.00% humidity, and *ad libitum* food and water. All procedures were performed on animals in accordance with the guidelines of the Ethics Committee of the Urmia University, Urmia, Iran (IR-UU-AEG-3/63).[19] The VitB1 provided from Rooyan Darou Co. (Tehran, Iran) and MTX manufactured by Koçak Pharmaceuticals Ltd. (Istanbul, Türkiye) were used in this study. Treatments in six groups (each with six mice) were as follows: Healthy control group was received 0.10 mL normal saline intraperitoneally (IP), sham group was received MTX (10.00 mg kg^{-1} *per* week; IP),[11] positive control group was received only VitB1 (100 mg kg^{-1} *per* day; IP), and three experimental groups were received MTX (10.00 mg kg^{-1} *per* week; IP) plus 25.00, 50.00, and 100 mg kg^{-1} *per* day; IP VitB1, respectively.[20] For malondialdehyde (MDA) and total antioxidant capacity (TAC) assessments, sperm quality and quantity evaluation, and *in vitro* fertilization (IVF), 36 days after treatments, the animals were euthanized with ketamine overdose (100 mg kg^{-1}), and their left testicles were dissected out and stored at – 70.00 ˚C until commence of aforementioned analyses.

Preparation of cauda epididymis's sperms. To extract sperm from the testes, the abdominal skin was sterilized with 70.00% ethanol. After opening the abdominal cavity, the connective tissues surrounding the testis were removed and the tail of each epididymis was separated from the testes and placed in sterile tubes containing 1.00 mL of human tubal fluid (HTF; Sigma-Aldrich, St. Louis, USA) containing bovine serum albumin (BSA; 4.00 mg mL^{-1}), previously incubated for equilibration. The sperms were incubated in a CO_2 incubator at 37.00 ˚ C. After 30 min, the sperms were released and dispersed in the culture medium (Origio, Måløv, Denmark).[21]

Sperm count. Ten microliters of diluted sperms (1:20) were placed on a Neubauer slide and the number of sperms observed by a light microscope was counted at 400 × magnification. The number of sperms was calculated according to the following formula:

$$Number\ of\ sperms = n \times 50{,}000 \times d$$

where, *n* is the number of sperms counted in five squares of the Neubauer slide and *d* is the inverse dilution of the suspension containing sperm (d = 20).

Sperm motility. Sperm mobility was evaluated according to the World Health Organization standard method for manual examination of sperm motility. Accordingly, sperm samples were diluted (1 : 8) in culture medium before testing. A volume of 20.00 µL of sperm sample was placed in the sperm study area and examined at 400× magnification. Only motile spermatozoa with forward progression were counted and recorded in 10 fields. Finally, mobility was evaluated according to the following method:[22]

$$Motility\ (\%) = motile\ sperm / motile + non\text{-}motile\ sperm \times 100$$

Sperm viability and morphology. Eosin-Nigrosin staining was used to distinguish viable sperms from non-viable ones. Dead spermatozoa are permeable to Eosin dye due to the plasma membrane damage. For this purpose, 20.00 µL of sperms were mixed with 20.00 µL of Eosin solution on the slide. After 30 sec, 20.00 µL of Nigrosin solution was added to this mixture. After proper incubation, sperm viability was checked using light

microscopy at 400× magnification. The percentage of Eosin-positive sperm in each sample was counted and the percentage of survival was extracted. Both Aniline Blue and Eosin-Nigrosin dyes were used to evaluate sperm morphology. Sperms looked abnormal with Aniline Blue staining were counted and the results were expressed as a percentage. In Eosin-Nigrosine staining, sperms contained cytoplasmic droplets were considered as immature sperms.[23]

Immature sperms. Aniline Blue staining was used to evaluate sperm nucleus maturity. In spermatogenesis, chromatin histones are replaced by protamine in the sperm nucleus. Immature sperms with histone residues are stained with Aniline Blue, being an important indicator of sperm maturity. Air-dried smears of sperm samples were fixed with 3.00% formalin for 30 min. Then, the slides were immersed in Aniline Blue (5 min). Alter that, the slides were washed with distilled water and examined with a light microscope at 400 × magnification. The percentages of mature sperms (pale) and immature sperms (grayish dark blue) were determined.[24]

Sperm DNA damage. Acridine Orange staining was used to assess any breaks in the double-stranded DNA of mouse sperm. Desired smears of sperms were prepared and dried, then fixed for 2 hr using Carnoy's solution, and stained with Acridine Orange (10 min). After washing the slides with water, they were examined with a fluorescence microscope (Nikon, Tokyo, Japan) by a 460 nm filter and the results were reported as percentage. Intact double-stranded DNA showed a green fluorescent, while denatured single-stranded DNA had a yellow to red color.[24]

***In vitro* fertilization.** After 36 days, mice were prepared for IVF and three mice from each group were considered. To stimulate ovulation, 10 units of pregnant mare's serum gonadotropin (Sigma-Aldrich) was IP injected into each female mouse. After 48 hr, 10 units of human chorionic gonadotropin (Sigma-Aldrich) was IP injected. Twelve to fourteen hr after human chorionic gonadotropin injection, female mice were euthanized, their fallopian tubes were removed, and the ampullae were dissected in a plastic container containing HTF medium plus 4.00 mg kg^{-1} BSA. The cumulus masses around the oocytes were separated and introduced into the HTF medium plus 4.00 mg kg^{-1} BSA. Fertilized sperm micro-drops (1×10^6 sperm *per* mL) were prepared in HTF plus 4.00 mg kg^{-1} BSA, and 10 - 15 oocytes were placed in each sperm micro-drop (150 µL). The fertilization process was carried out by incubation for 4 to 6 hr at 37.00 ˚C under 5.00% CO_2. Then, denuded fertilized eggs were transferred into the fresh drops of HTF plus 4.00 mg kg^{-1} BSA for embryo culture. All medium drops were covered with mineral oil (Sigma-Aldrich), and fertilized oocytes were evaluated for the appearance of pronuclei under an inverted microscope at 200× magnification (IX70; Olympus, Tokyo, Japan). Then, the zygotes were washed three times with Potassium Simplex Optimized Medium (KSOM), transferred to fresh KSOM, and cultured for another five days at 37.00 ˚C under 5.00% CO_2. After 24 hr, the number of two-cell embryos was evaluated *in vitro*; the blastocyst stage was monitored on the 3rd day.[25]

Malondialdehyde level. To determine the extent of lipid peroxidation, the MDA content of the collected testis samples was measured using the thiobarbituric acid (TBA; Sigma-Aldrich) reaction.[26] Briefly, 0.30 - 0.40 g of testicular tissue was homogenized in ice-cold KCl (150 mM; Merck, Darmstadt, Germany) and then, the mixture was centrifuged at 3,000 *g* for 10 min. After that, 0.50 mL of the supernatant was mixed with 3.00 mL of 1.00% phosphoric acid (Merck) and after vortex mixing, 2.00 mL Lof 6.70 g L^{-1} thiobarbituric acid was added to the samples. The samples were heated at 100 ˚C for 45 min and then cooled on ice. After adding 3.00 mL of N-butanol (Merck), the samples were centrifuged again at 3,000 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm wavelength with spectrophotometer (Epoch Biotek, Winooski, USA), and the MDA concentration was prepared based on the calibration curves and calculated using MDA standards. The amount of MDA was expressed as nmol *per* mg of protein.[27]

Total antioxidant capacity (TAC) level. The evaluation of TAC was performed using the iron reduction antioxidant power assay.[9] Briefly, at acidic pH, which was created using acetate buffer (300 mM; pH: 3.60), the reduction of Fe^{III}-2, 4, 6-tri-2-pyridyl-1, 3, 5-triazin (Merck) to the ferrous form results in deep blue color, being measured at 593 nm. Aqueous solution of $FeSO_4.7H_2O$ (Merck) was used as a blank solution and freshly prepared ascorbic acid (Sigma-Aldrich) was used as a standard solution.[28]

Statistical analysis. Statistical analysis was performed using SPSS Software (version 21.0; IBM Corp., Armonk, USA). Finally, sperm analysis and IVF data were compared between all groups and analyzed using one-way analysis of variance followed by Bonferroni's *post hoc* test. The *p*-value less than 0.05 was considered significant. All data are presented as mean ± standard error.

Results

Biochemical findings. The MDA level was increased in the MTX group in comparison with the control group ($p < 0.05$); whereas, in the groups receiving vitamin $VitB_1$ with MTX, its level was decreased in proportion to the increase in the $VitB_1$ doses. Conversely, TAC level of the testicular tissue was higher in control and $VitB_1$ groups in comparison with the MTX group ($p < 0.05$); whereas, the highest level of TAC was observed in the group received maximum dose of $VitB_1$ (Fig. 1).

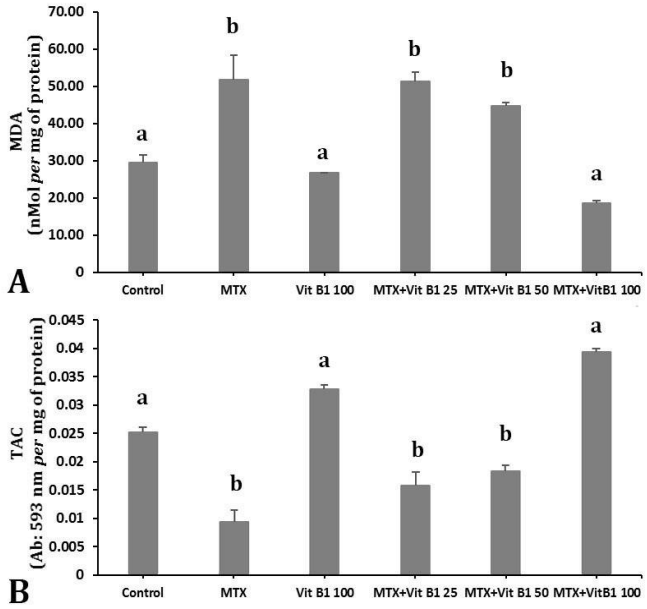

Fig. 1. A) Malondialdehyde (MDA) and **B)** total antioxidant capacity (TAC) levels in the testicular tissue of all experimental groups. The highest and lowest values of MDA and TAC were observed in methotrexate (MTX) group, respectively. While, in the experimental groups, MDA was decreased and TAC increased depending on the thiamine (VitB1) dose.

[ab] Different letters indicate significant differences between groups ($p < 0.05$).

Sperm count. The mean number of sperm in the MTX group showed a significant decrease compared to the group receiving VitB1 and control group ($p < 0.05$). In VitB1 100 mg kg^{-1} + MTX group, there was a significant increase in sperm count compared to the sham group ($p < 0.05$; Table 1).

Sperm motility and viability. The percentage of sperm motility and the number of live sperms in the MTX group showed a significant decrease in comparison with the VitB1 and control groups ($p < 0.05$). In the VitB1 + MTX groups, an increase in the percentages of sperm motility and viability was seen. However, only the VitB1 50.00 and 100 mg kg^{-1} + MTX groups showed a significant increase in comparison with the MTX group ($p < 0.05$; Table 1 and Fig. 2).

Immature sperms. The percentage of immature sperms in the MTX group was significantly increased in comparison with the VitB1 and control groups. Whereas, despite the decrease in the rate of the immature sperms in VitB1 25.00, 50.00, and 100 mg kg^{-1} + MTX groups, there were no significant differences compared to the MTX group (Table 1 and Fig. 2).

Sperm DNA damage. The sperms with DNA damage in the MTX group showed a significant increase compared to the VitB1 and control groups. But, despite the decrease in the percentages of this parameter in the VitB1 25.00, 50.00, and 100 mg kg^{-1} + MTX groups, this reduction was not significant in comparison with the sham group (Table 1 and Fig. 2).

Fertilization. Fertilization rate was reduced in MTX group compared to the control and VitB1 groups ($p < 0.05$)**.** The groups received vitamin along with MTX showed an increase in the percentage of fertilization; but this increase was only significant in the VitB1 50.00 and 100 mg kg^{-1} + MTX groups compared to the sham group ($p < 0.05$; Table 2 and Fig. 3).

Morulae and two-cell embryos. The percentages of morulae and two-cell embryos were decreased in the MTX group; but they did not show a significant difference compared to the control and VitB1 groups**.** There was an increase in the percentages of two-cell embryos and morulae in VitB1 50.00 and 100 mg kg^{-1} + MTX groups; but this rise did not show a significant difference compared to the sham group (Table 2 and Fig. 3).

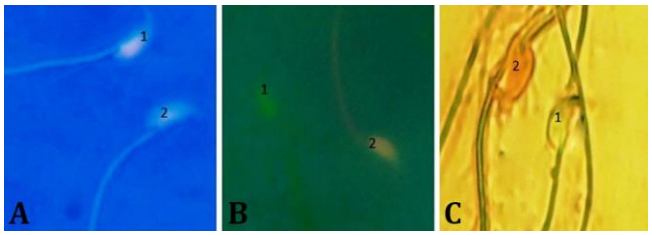

Fig. 2. Sperm maturity, DNA damage, and viability analyses (1,000×). **A)** Aniline Blue staining revealed the mature sperm with a colorless head (1) and immature sperm with a blue head (2), **B)** Acridine Orange staining indicated the healthy DNA sperm with a green head (1) and DNA-damaged sperm with an orange head (2), and **C)** Eosin-Nigrosin staining showed the live sperm with a pale head (1) and dead sperm with a red head (2).

Table 1. Sperm parameters in all experimental groups.

Groups	Sperm count (× 10^6 *per* mL)	Sperm motility (%)	Sperm viability (%)	Immature sperms (%)	DNA-damaged sperms (%)
Control	52.00 ± 2.30[a]	86.00 ± 2.08[a]	87.66 ± 2.33[a]	4.66 ± 0.88[a]	7.33 ± 1.76[a]
MTX	33.66 ± 2.02[b]	58.00 ± 5.50[b]	60.66 ± 1.45[b]	13.00 ± 2.08[b]	20.66 ± 0.88[b]
VitB1	53.33 ± 3.84[a]	88.33 ± 4.70[a]	89.66 ± 2.02[a]	3.66 ± 1.20[a]	6.00 ± 0.57[a]
MTX + VitB1 25.00 mg kg^{-1}	38.33 ± 1.45[b]	63.33 ± 2.33[b]	67.33 ± 1.45[b]	10.00 ± 0.57[b]	20.33 ± 1.20[b]
MTX + VitB1 50.00 mg kg^{-1}	44.66 ± 2.33[b]	78.00 ± 2.64[c]	79.00 ± 1.73[c]	8.00 ± 1.15[b]	15.33 ± 0.88[b]
MTX + Vit B1 100 mg kg^{-1}	48.00 ± 1.73[c]	80.00 ± 0.57[c]	80.33 ± 2.72[c]	6.66 ± 1.20[b]	17.28 ± 2.47[b]

MTX: Methotrexate; VitB1: Thiamine.

[abc] Different superscript letters indicate significant differences in each column between different groups ($p < 0.05$).

Table 2. *In vitro* fertilization outcomes in all experimental groups.

Groups	Fertilization rate (%)	Two-cell embryo (%)	Morula (%)	Blastocyst (%)	Hatched embryo (%)	Arrested embryo (%)
Control	81.66 ± 2.73[a]	71.99 ± 1.98[a]	61.03 ± 3.50[a]	57.69 ± 3.97[a]	52.51 ± 3.73[a]	25.89 ± 3.26[a]
MTX	45.24 ± 1.18[b]	60.89 ± 3.42[a]	49.48 ± 1.97[a]	35.29 ± 3.75[b]	25.26 ± 3.36[b]	58.14 ± 6.31[b]
VitB1	79.77 ± 3.60[a]	77.27 ± 1.15[a]	63.80 ± 4.03[a]	59.89 ± 3.76[a]	50.06 ± 2.30[a]	35.13 ± 2.19[a]
MTX + VitB1 25.00 mg kg^{-1}	49.38 ± 2.55[b]	58.19 ± 5.68[a]	47.29 ± 2.91[a]	39.16 ± 4.73[b]	31.47 ± 2.61[b]	45.45 ± 4.54[b]
MTX + VitB1 50.00 mg kg^{-1}	63.82 ± 2.87[c]	67.47 ± 5.16[a]	55.97 ± 6.53[a]	47.86 ± 3.78[b]	44.00 ± 4.35[c]	39.24 ± 6.14[b]
MTX + VitB1 100 mg kg^{-1}	67.02 ± 3.20[c]	66.03 ± 4.07[a]	58.03 ± 4.40[a]	55.92 ± 3.52[c]	50.24 ± 2.65[c]	23.80 ± 1.19[c]

MTX: Methotrexate; VitB1: Thiamine.

[abc] Different superscript letters indicate significant differences in each column between different groups ($p < 0.05$).

Blastocysts. The percentage of blastocyst stage embryos in the MTX group was decreased compared to the control and Vit B1 groups ($p < 0.05$). The VitB1 + MTX groups showed an increase in the percentage of blastocysts; but this increase was only significant in the VitB1 100 mg kg^{-1} + MTX group compared to the sham group ($p < 0.05$; Table 2 and Fig. 3).

Hatched embryos. The percentage of hatched embryos in the MTX group was decreased and showed a significant difference compared to the control and VitB1 groups ($p < 0.05$). Also, there was a significant increase in hatched embryos percentage in VitB1 50.00 and 100 mg kg^{-1} + MTX groups compared to the sham group ($p < 0.05$; Table 2 and Fig. 3).

Arrested embryos. The percentage of arrested embryos in the MTX group was increased significantly compared to the control and VitB1 groups ($p < 0.05$). In the VitB1 plus MTX groups, there was a decrease in the percentage of arrested embryos; but this reduction showed a significant difference only in the VitB1 100 mg kg^{-1} + MTX group compared to the MTX group ($p < 0.05$; Table 2 and Fig. 3).

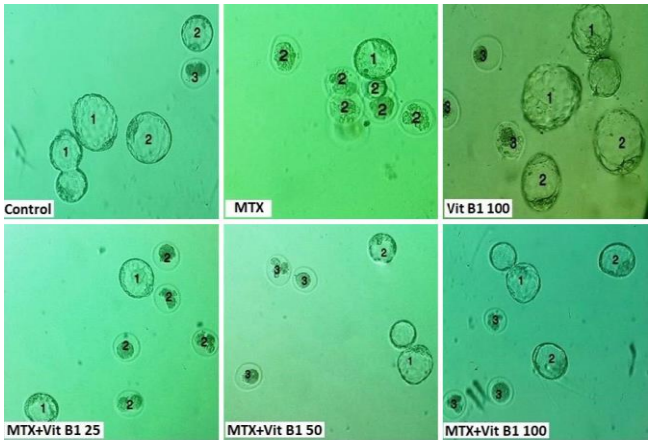

Fig. 3. Different stages of *in vitro* embryo development. 1: Hatched embryo, 2: Blastocyst, and 3: Arrested embryo (200×).

Discussion

According to results of this study, an increase in MDA levels was seen in testicular tissue in the MTX group compared to the control groups. However, in the groups receiving VitB1, the quantity of MDA was decreased proportionally with the increase in vitamin dose. Furthermore, TAC levels in the control and VitB1 groups were increased compared to the MTX group. According to the results of previous studies, the MTX increases inflammation and impairs energy sources in the germ cell lineage.[26] The evaluation of sperm quality revealed the damage caused by MTX in the sham group, which confirmed a significant decrease in the mean concentration of sperm and sperm motility and viability compared to the control group. Whereas, in the groups receiving VitB1 along with MTX, the amount of these parameters increased. Decreased sperm count observed in the current study more likely is the result of oxidative stress, that is completely in accordance with previous reports.[29] According to the results of a study, MTX can diminish DNA integrity in sperm and cause damages *via* oxidative stress.[30] The spermatogenic cells play an important role in regulating of the testosterone hormone, consequently it can be *said* that, the use of MTX increases the production of free radicals, which in turn reduces the rate of spermatogenesis and ultimately leads to a decrease in the number of sperms.[31] A large number of mitochondria in sperm indicates the high energy requirement of sperm for motility. In the patients receiving MTX, the upsurge of lipid peroxidation in mitochondria leads to the destruction of the mitochondrial membrane.[32] Thiamine acts as a cofactor and energy source for many cellular enzymes and also improves antioxidant levels by participating in the pentose phosphate cycle that produces nicotinamide adenine dinucleotide phosphate.[18] On the other hand, through these mechanisms, thiamine can stop the reduction of sperm motility and also increase its ability to move fast. It has been reported that thiamine pyrophosphate may act as an agent that ameliorates rat's testicular damage induced by cisplatin in a dose dependent way. The proposed mechanism might be attributed to its antioxidant and anti-apoptotic roles.[21] However, most likely retro-protective effect of thiamine is through protecting proteins, lipids, and DNA against free radicals and regulation of *the* glucose metabolism. The results of this investigation revealed that oxidative stress caused by MTX in the sham group triggered a significant increase in

immature (low quality chromatin) and DNA-damaged spermatozoa compared to the MTX + VitB1 groups. In the spermiogenesis stage, in the chromosomes of newly produced sperms, the histones attached to the DNA strands are replaced by protamine proteins; as a result of this process, the chromatin is condensed and the copying process is stopped. In the stage of epididymal maturation, thiol groups of protamine are oxidized and disulfide bands are formed in the protein structure. The existence of these disulfide bridges is critical for the compaction and physical/chemical stability of chromatin. For optimal chromatin condensation, nucleoprotein surrounds double-stranded DNA, acting as a protective factor against various destructive issues, such as acids, heat, proteases, DNAase, free radicals, and detergents. Any disturbance in the chromatin density will be associated with subsequent DNA and nuclear damage**.** Most of these damages occur as a result of disruption in the middle stage of spermiogenesis and replacement of histone by protamine. In general, the three major etiologies of sperm DNA damage are inadequate and inappropriate DNA condensation during maturation, oxidative stress, and apoptosis.[32-35] Methotrexate increases the production of ROS, and investigations have also shown that increases in the production of free radicals cause oxidative damage to mitochondrial membrane;[36] as stated before, thiamine also plays a preventive role against oxidative stress. This results completely comply with previous studies. *In vitro* fertilization rate in the sham group was significantly lower than control groups. However, in the MTX + VitB1 groups, this trend was stopped and reversed, indicating the protective effect of VitB1 against the oxidative stress affecting the rate of IVF.

Regarding the rates of blastocysts formation and their hatching, our study revealed that the average number of blastocysts resulting from IVF in the groups receiving MTX had a significant decrease compared to the control groups due to the oxidative stress; however, the use of VitB1 along with MTX caused compensatory effects. The improvement of sperm quality by administration of thiamine may lead to improvement of the IVF outcomes. It has been proven that sperm selection can be improved through one or more factors, including sperm genetic integrity, fertilization capacity, embryo production, and *in vitro* survival, as well as pregnancy and delivery rates following IVF and intra-cytoplasmic sperm injection.[37]

On the other hand, the presence of higher amounts of ROS leads to sperm abnormalities and infertility. The overwhelming data have proven that oxidative stress in sperm has an impact not only on DNA integrity but also on the dynamics of epigenetic reprogramming, which may harm the paternal genetic and epigenetic contribution to the developing embryo and affect embryo development and its quality;[38] therefore, this oxidative damage will be the main cause of sperm dysfunction.[39]

In the compensatory way, antioxidant defense mechanisms in the testis are important to protect sperms against ROS. According to the results of a former study, kisspeptin-10 (Kiss), a potential antioxidant agent, treatment mitigates MTX-induced reproductive toxicity.[40] In this study, it was assumed that VitB1 played such a role. Likewise, it was found in the present study that the number of arrested embryos had a significant increase in the MTX group in comparison with the control groups due to the oxidative stress, and the use of VitB1 with MTX reduced this destructive effect in a dose-dependent manner. As mentioned earlier, in the process of sperm nuclear maturation, the main factor for the integrity of DNA strands is the replacement of protamine proteins, and if this process is disrupted by oxidizing agents, DNA will be significantly damaged.[41] On the other hand, embryos fertilized with these sperms (with broken DNA strands) produce low-quality and/or arrested embryos.[42] This study had limitations regarding the sample size and lack of direct measurement of oxidative stress and apoptosis.

In conclusion, the present study revealed that oxidative stress is a major side effect of MTX, inducing testicular damage and lowering sperm quality, and thiamine preserves fertility through having positive effects on various fertility indices *via* oxidative stress suppression. Additional research with a wider scope will be needed, especially by focusing on the role of thiamine in the cell energy cycle.

Acknowledgments

All authors wish to specially thank the Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, for scientific helps.

Conflict of interest

The current manuscript is a part of the research being done as a doctoral course thesis, registered in the Deputy Committee of Urmia University, Urmia, Iran.

References

1. DeVita VT Jr, Chu E. A history of cancer chemotherapy. Cancer Res 2008; 68(21): 8643-8653.
2. Vakalopoulos I, Dimou P, Anagnostou I, et al. Impact of cancer and cancer treatment on male fertility. Hormones (Athens) 2015; 14(4): 579-589.
3. Trost LW, Brannigan RE. Oncofertility and the male cancer patient. Curr Treat Options Oncol 2012; 13(2): 146-160.
4. Tousson E, Bayomy MF, Ahmed AA. Rosemary extract modulates fertility potential, DNA fragmentation, injury, KI67 and P53 alterations induced by etoposide in rat testes. Biomed Pharmacother 2018; 98: 769-774.

5. Tousson E, Hafez E, Zaki S, et al. The cardioprotective effects of L-carnitine on rat cardiac injury, apoptosis, and oxidative stress caused by amethopterin. Environ Sci Pollut Res Int 2016; 23(20): 20600-20608.
6. Dabaja AA, Schlegel PN. Medical treatment of male infertility. Transl Androl Urol 2014; 3(1): 9-16.
7. Lotti F, Corona G, Mondaini N, et al. Seminal, clinical and colour-Doppler ultrasound correlations of prostatitis-like symptoms in males of infertile couples. Andrology 2014; 2(1): 30-41.
8. Arkalı G, Acısu TC, Badıllı N, et al. Effect of *Boswellia serrata* extract on methotrexate induced testicular damage. Revista Científica FCV-LUZ 2024; 34(1): e34289. doi: 10.52973/rcfcv-e34289.
9. Widemann BC, Adamson PC. Understanding and managing methotrexate nephrotoxicity. Oncologist 2006; 11(6): 694-703.
10. Liu M, Hales BF, Robaire B. Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. Biol Reprod 2014; 90(4): 72. doi: 10.1095/biolreprod.114.117754.
11. Padmanabhan S, Tripathi DN, Vikram A, et al. Cytotoxic and genotoxic effects of methotrexate in germ cells of male Swiss mice. Mutat Res 2008; 655(1-2): 59-67.
12. Armagan A, Uzar E, Uz E, et al. Caffeic acid phenethyl ester modulates methotrexate-induced oxidative stress in testes of rat. Hum Exp Toxicol 2008; 27(7): 547-552.
13. Makker K, Agarwal A, Sharma R. Oxidative stress & male infertility. Indian J Med Res 2009; 129(4): 357-367.
14. Hosen MB, Islam MR, Begum F, et al. Oxidative stress induced sperm DNA damage, a possible reason for male infertility. Iran J Reprod Med 2015; 13(9): 525-532.
15. De Luca M, Ciampo E, Rossi A. Study of the seminal fluid in subjects treated with methotrexate [Italian]. G Ital Dermatol Minerva Dermatol 1971; 46(6): 247-249.
16. Bomser J, Madhavi DL, Singletary K, et al. *In vitro* anticancer activity of fruit extracts from *Vaccinium* species. Planta Med 1996; 62(3): 212-216.
17. Marriott BP, Birt DF, Stalling VA, et al. Present knowledge in nutrition: basic nutrition and metabolism. 11th ed. New York, USA: Elsevier Inc. Academic Press; 2020; 225-230.
18. Friedrich W. Thiamin (vitamin B1, aneurin). Handbook of vitamins [German]. Munich, Germany: Urban & Schwarzenberg 1987; 240-258.
19. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16(2): 109-110.
20. Mnati MA, Mshimesh BA, Ibraheem MM, et al. Ameliorative effect of thiamine pyrophosphate against cisplatin-induced reproductive system damage within male rats. Sys Rev Pharm 2020; 11(11): 396-403.
21. Bakhtiary Z, Shahrooz R, Ahmadi A, et al. Protective effect of crocin on DNA damage of sperm and *in vitro* fertilization (IVF) in adult male mice treated with cyclophosphamide [Persian]. J Mazandaran Univ Med Sci 2014; 24(118): 49-59.
22. WHO. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. World health Organization. Cambridge, UK. Cambridge University Press; 1999.
23. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21(6): 776-798.
24. Sadeghi MR, Hodjat M, Lakpour N, et al. Effects of sperm chromatin integrity on fertilization rate and embryo quality following intracytoplasmic sperm injection. Avicenna J Med Biotechnol 2009; 1(3): 173-180.
25. Anbara H, Shahrooz R, Razi M, et al. The effect of vitamin C on mice hemolytic anemia induced by phenylhydrazine: an animal model study using histological changes in testis, pre-implantation embryo development, and biochemical changes. Iran J Basic Med Sci 2018; 21(7): 668-677.
26. Zarei L, Shahrooz R. Protective effects of *Cornus mas* fruit extract on methotrexate-induced alterations in mice testicular tissue: evidences for histochemical and histomorphometrical changes in an animal model study. Vet Res Forum 2019; 10(4): 307-313.
27. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-275.
28. Bonyadi F, Hasanzadeh S, Malekinejad H. Cyclopiazonic acid induced p53-dependent apoptosis in the testis of mice: another male related risk factor of infertility. Environ Toxicol 2021; 36(5): 903-913.
29. Afkhami-Ardakani M, Hasanzadeh S, Shahrooz R, et al. Antioxidant effects of *Spirulina platensis* (*Arthrospira platensis*) on cyclophosphamide-induced testicular injury in rats. Vet Res Forum 2018; 9(1): 35-41
30. Ley D, Jones J, Parrish J, et al. Methotrexate reduces DNA integrity in sperm from men with inflammatory bowel disease. Gastroenterology 2018; 154(8): 2064-2067.
31. Jensen SB, Mouridsen HT, Reibel J, et al. Adjuvant chemotherapy in breast cancer patients induces temporary salivary gland hypofunction. Oral Oncol 2008; 44(2): 162-173.
32. Tabassum H, Parvez S, Pasha ST, et al. Protective effect of lipoic acid against methotrexate-induced oxidative stress in liver mitochondria. Food Chem Toxicol 2010; 48(7): 1973-1979.
33. Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to *in situ* denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. J Androl

1995; 16(1): 80-87.
34. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003; 79(4): 829-843.
35. Sakkas D, Mariethoz E, Manicardi G, et al. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 1999; 4(1): 31-37.
36. Calabrese V, Lodi R, Tonon C, et al. Oxidative stress, mitochondrial dysfunction and cellular stress response in Friedreich's ataxia. J Neurol Sci 2005; 233(1-2): 145-162.
37. Oseguera-López I, Ruiz-Díaz S, Ramos-Ibeas P, et al. Novel techniques of sperm selection for improving IVF and ICSI outcomes. Front Cell Dev Biol 2019; 7: 298. doi: 10.3389/fcell.2019.00298.
38. Wyck S, Herrera C, Requena CE, et al. Oxidative stress in sperm affects the epigenetic reprogramming in early embryonic development. Epigenetics Chromatin 2018; 11(1): 60. doi: 10.1186/s13072-018-0224-y.
39. Moustafa MH, Sharma RK, Thornton J, et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 2004; 19(1): 129-138.
40. Güvenç M, Aksakal M. Ameliorating effect of kisspeptin-10 on methotrexate-induced sperm damages and testicular oxidative stress in rats. Andrologia 2018; 50(8): e13057. doi: 10.1111/and.13057.
41. Koskimies AI, Savander M, Ann-Marie N, et al. Sperm DNA damage and male infertility [Finnish]. Duodecim 2010; 126(24): 2837-2842.
42. Avendaño C, Franchi A, Duran H, et al. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. Fertil Steril 2010; 94(2): 549-557.