Ann Med Res

Current issue list available at AnnMedRes

Annals of Medical Research

journal page: www.annalsmedres.org

Assessment of the effects of two different doses of methotrexate in rat ovarian tissue: A histological study

Betul Yalcin[a,*], Kubra Tugce Kalkan[b], Eda Koseoglu[c], Enes Karaman[d], Arzu Yay[c]

[a] *Adıyaman University, Faculty of Medicine, Department of Histology and Embryology, Adıyaman, Türkiye*
[b] *Kırşehir Ahi Evran University, Faculty of Medicine, Department of Histology and Embryology, Kırşehir, Türkiye*
[c] *Erciyes University, Faculty of Medicine, Department of Histology and Embryology, Kayseri, Türkiye*
[d] *Niğde Ömer Halisdemir University, Faculty of Medicine, Department of Gynecology and Obstetrics, Niğde, Türkiye*

ARTICLE INFO

Keywords:
Anti-Mullerian hormone
Methotrexate
Ovarian reserve

Received: May 29, 2024
Accepted: Aug 15, 2024
Available Online: 28.08.2024

DOI:
10.5455/annalsmedres.2024.05.098

Abstract

Aim: The use of methotrexate (MTH), a drug commonly prescribed to treat rheumatoid arthritis, cancer, and certain autoimmune diseases, has been linked to early ovarian failure and infertility in women when used in varying amounts and durations. In this study, the possible effects of MTH exposure at different doses on rat ovary were investigated histologically and immunohistochemically.

Materials and Methods: A total of 21 female rats, Wistar albino, were utilized in the investigation. The control group (n = 7) was not subjected to any treatment. The 10 mg/kg MTH group (n = 7) and the 20 mg/kg MTH group (n = 7) were given intraperitoneal injections of 10 mg/kg and 20 mg/kg methotrexate, respectively. Five days after injection, the ovarian tissues were taken out of the anesthetized rats and subjected to routine histological tissue processing. The histological evaluation was performed with hematoxylin and eosin (H&E) and Masson's trichrome (MT) on paraffin block sections. Additionally, immunohistochemical staining was performed to determine AMH immunoreactivity.

Results: In both MTH groups, histopathological changes were observed, particularly in the 20 mg/kg group, and these included the presence of areas of edema as well as dilated or congested blood vessels. Furthermore, the 20 mg/kg MTH group exhibited increased fibrotic tissue in the ovarian medulla. The number of follicles and AMH immunoreactivity were observed to be reduced in the MTH groups, with the reduction reaching a statistically significant level in the 20 mg/kg MTH group ovary. In terms of AMH immunoreactivity, it is demonstrated that there is no statistical significance between the control and 10 mg/kg MTH groups.

Conclusion: The dose of MTH should be considered carefully, as increasing the dose may lead to deterioration of ovarian histopathology and a reduction in the reserve of the ovary.

Introduction

MTH, a folic acid antagonist, exerts its pharmacological effect by inhibiting the activity of the dihydrofolate reductase enzyme. The synthesis of pyrimidines, purines, and nucleic acids is inhibited when MTH binds to this enzyme [1]. This substance has the potential to inhibit the proliferation and development of malignant and some non-cancerous cells. Furthermore, it may also induce toxic effects, particularly in cells with a high capacity to divide [2,3]. MTH has been shown to arrest cells in metaphase in animal studies [3]. The drug is used to treat malignant tumors, cancer, autoimmune, and inflammatory diseases due to its anti-proliferative, anti- inflammatory, and immunosuppressive effects [4,5]. MTH is effective but has a high efficacy/toxicity ratio, which may lead to multi-organ toxicity due to its antimetabolic and cytotoxic effects [6].

MTH-induced organ and tissue damage is defined by the generation of reactive oxygen species, oxidative stress, and inflammation, particularly in response to high doses or long-term use of MTH. Such damage can result in functional and structural abnormalities of the ovaries, with the potential for infertility in high doses [7,8]. The measurement of anti-Mullerian hormone (AMH) has emerged as a sensitive and reliable biomarker for the evaluation of ovarian reserve following gonadotoxic treatment, includ-

*Corresponding author:
Email address: byalcin@adiyaman.edu.tr (Betul Yalcin)

ing chemotherapeutic, radiotherapeutic, and surgical interventions. AMH is a glycoprotein differentiation factor that belongs to the TGF-β family, which includes inhibin and activin [9]. This hormone is secreted from preantral and antral follicles' granulosa cells independently of the menstrual cycle. Serum levels of AMH can be used to predict potential impacts on the ovarian reserve [10,11].

In conclusion, the goal of this investigation was to determine the histopathological changes generated by two different doses of MTH in rat ovarian tissue and its effects on ovarian reserve histologically and immunohistochemically.

Materials and Methods

Preparation of MTH

MTH at a concentration of 50 mg/mL was acquired by Kocak Pharma (Istanbul, Turkey).

Animal care

The Erciyes University Local Ethics Committee granted permission (decision number 23/081) to obtain twenty one female adult Wistar albino rats. for the current study. The animal experiment for this study was performed at the Erciyes University Experimental Research, Application, and Research Center. The rats were placed in sterilized cages with unlimited access to pellets and water and subjected to a 12-hour light/dark period.

Experimental design

The results of the literature review indicated that 10 mg/kg and 20 mg/kg MTH administration would be appropriate for inclusion in the present study. The study consisted of three groups, each comprising seven animals. The procedures to be performed on each group are outlined below: Control group; no treatment was performed. 10 mg/kg Methotrexate group; on the first day of the experiment, 10 mg/kg MTH was applied intraperitoneally in one dosage [12]. 20 mg/kg Methotrexate group; on the first day of the experiment, 20 mg/kg MTH was applied intraperitoneally in one dosage [13,14].

Five days following the dosage, all animals were anesthetized with a combination of xylazine and ketamine. For histological and immunohistochemical analyses, rat ovarian tissue was extracted.

Histopathological examination

Following the conclusion of the experiment, the entire ovarian tissues from each experimental group were obtained and preserved in 10% formaldehyde. The tissues were taken out of the fixative and rinsed with tap water following a 72-hour incubation period in formaldehyde. Dehydration was achieved by passing the sample through a series of gradually increasing alcohol solutions, followed by transparency achieved through three different xylene solutions. Finally, the paraffin-embedded tissues were prepared for sectioning, and 5 µm-thick 1/12 serial sections were taken from each prepared paraffin block onto slides [15-17]. Subsequently, the deparaffinization and rehydration of the sections were conducted prior to staining them with hematoxylin and eosin (H&E) and Masson's trichrome dyes. The stained sections were subjected to a series of increasingly dilute alcohols and xylol solutions in order to facilitate covering. Visualization and photography of the sections were conducted using an Olympus BX51 (Olympus Corp., Tokyo, Japan) light microscope.

Morphological classification and counting of follicles

Serial sections of ovarian tissues from all experimental groups were examined under light microscopy to quantify follicular capacity using H&E staining. Only follicles with oocytes containing the nucleus and nucleolus were included in the counting. All follicles in the sections were counted based on the predetermined criteria. The follicle types were defined as primordial, primary, preantral, secondary, tertiary, and atretic follicles, according to the shape and number of rows of granulosa cells around the oocyte and the presence or absence of an antrum within the follicle. Primordial follicle; oocyte enclosed by a single layer of flat granulosa cells, primary follicle; oocyte enclosed by a single layer of cuboidal granulosa cells, preantral follicle; oocyte enclosed by more than one layer of cuboidal granulosa cells, secondary follicle; follicles with antral spaces between multilayered granulosa cells, tertiary follicle; follicle consisting of a large cavity and clusters of cells called corona radiata and coumulus oophorus extending into this cavity, atretic follicle; follicles formed by disruption of the shape and organization of oocytes and granulosa cells. Healthy follicles, which were classified and counted, retained a continuous basement membrane, germinal epithelium, and oocyte integrity, whereas these structures were absent in atretic follicles. The whole number of follicles in each rat ovary was counted using the previously stated parameters [16].

Immunohistochemistry analysis

The anti-Mullerian hormone (AMH) immunoreactivity in ovarian tissue was determined by means of the Avidin-Biotin-Peroxidase labeling procedure. The sections were initially deparaffinized with xylene and subsequently hydrated with a decreasing alcohol series. The sections were then washed with deionized water before being subjected to heat in a microwave oven for 20 minutes at 95°C in a 0.01 M sodium citrate buffer (pH 6.0). After the sections had remained in the same solution for 20 minutes at room temperature, they were subjected to a wash with phosphate-buffered saline (PBS). The application of 3% hydrogen peroxide (H_2O_2) for a period of ten minutes was employed to block the endogenous peroxidase activity. The following were performed in a humid chamber, with the staining kit employed in accordance with the instructions provided by the producer. In order to cover areas beyond the antigenic regions, Ultra V Block was applied to the sections for a period of 10 minutes. Following an overnight treatment at 4°C with the AMH (Santa Cruz, Sc166752) primer antibody, PBS washing was applied to the sections. Biotinylated secondary antibodies were then applied to them for ten minutes. Subsequent to the incubation in PBS, the tissue sections were subjected to a peroxidase-conjugated streptavidin treatment. Tetrahydrochloride was employed to demonstrate the presence of immunoreactivity, and Gill haematoxylin was utilized for

nuclear staining. Before being cleaned in xylene and coverslipped in entellan, the sections were dehydrated in higher concentrations of alcohol.

Images were obtained using the Olympus BX51 light microscope. Ten microscopic fields were evaluated for each preparation with the Image J program [16,17].

Statistical analysis

The obtained data was subjected to normality testing using the Shapiro-Wilk and Kolmogorov- Smirnov tests. The data for the number of ovarian follikul and AMH immunoreactivity for the preantral and secondary follicles, which exhibited a normal distribution, were subjected to a post hoc Tukey analysis and an analysis of variance (ANOVA) and presented as the mean ± SE. The data for AMH immunoreactivity for the primary follicles, which was not normally distributed, was subjected to a Kruskal-Wallis test and a Tukey post-hoc test and presented as the median (quartile 1-quartile 3). The analysis of the data was done by the GraphPad Prism program (version 9.0, GraphPad Inc., San Diego, CA). All analyses were assessed at a 95% level of confidence ($p<0.05$).

Results

Histopathological results

Under light microscopy, H&E-stained ovarian sections were examined to assess ovarian morphology and ascertain whether MTH had an influence on the ovarian follicle and ovarian tissue. In the control group, ovarian tissues were observed to maintain their normal histomorphological characteristics. Both the cortex, consisting of follicles at various stages of growth, and the medulla, consisting of loose connective tissue, retained their typical histological appearance. In comparison to the control group, the MTH-treated rats ovaries showed a decrease in follicles and an increase in dilated or congested blood vessels in the medulla. In addition to the presence of edema, the findings were more noticeable in the 20 mg/kg MTH group (Figure 1).

To analyze how MTH influenced the morphological composition of connective tissue and the accumulation of collagen fibers in formalin-fixed ovarian sections, they were stained with MT. In the 10 mg/kg MTH group, there was no detectable increase in cortical or medullary fibrosis compared to the control group. However, the 20 mg/kg MTH group exhibited the highest amount of fibrosis in the medulla (Figure 1).

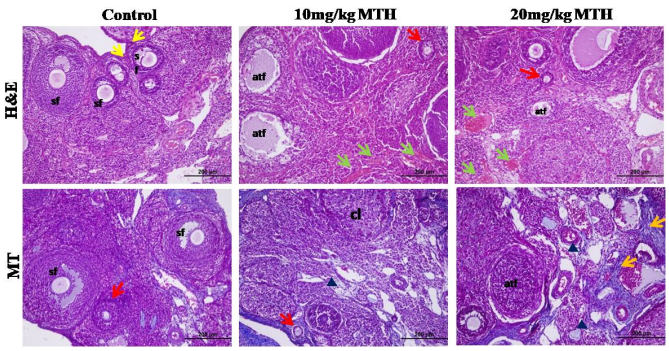

Figure 1. H&E and MT stained images in rat ovarian sections from each experimental group. Primordial follicles (yellow arrow), primary follicles (red arrow), secondary follicle (sf), corpora lutea (CL) and atretic follicles (AF). Congested blood vessels (green arrow), and edema (arrow head). HE: Hematoxylin-Eosin staining, 200x. Fibrosis (orange arrow). MT: Masson's Trichrome staining, 200x.

MTH induced changes in ovarian follicular reserve

To ascertain the stage of ovarian follicular development at which MTH is effective, primordial, primary, secondary, antral, and atretic follicles were taken into account in every 12 stained sections and shown in Table 1. The quantity of all follicular types demonstrated a reduction in both MTH-treated groups. The primordial, primary, and preantral follicle counts in the 10 mg/kg MTH group were comparable to those in the control group ($p>0.05$). In contrast to the control group, the group treated with 10 mg/kg of MTH demonstrated a significant reduction in the number of secondary and tertiary follicles, which represent the later stages of follicle development ($p<0.05$). In particular, a significantly lower number of all follicular types was observed in the 20 mg/kg MTH group relative to the control group ($p<0.05$). The only variable in which the two MTH groups differed statistically was the number of secondary follicles ($p<0.05$). Furthermore, compared to the control group, the MTH groups had a considerably larger number of atretic follicles. Moreover, follicles in the MTH group exhibited significantly higher levels of atresia than those in the control group ($p<0.05$).

Table 1. Number of follicles in different stages of ovarian follicular development.

Follicle type	Control	10 mg/kg MTH	20 mg/kg MTH	p
Primordial	37.80 ± 4.817^{a}	33.20 ± 5.848^{ab}	25.80 ± 3.564^{b}	0.007
Primary	19.40 ± 4.278^{a}	16.60 ± 2.302^{ab}	12.00 ± 2.236^{b}	0.008
Preantral	14.00 ± 2.915^{a}	11.80 ± 3.114^{ab}	8.40 ± 2.074^{b}	0.022
Secondary	17.40 ± 2.302^{a}	12.80 ± 1.924^{b}	8.4 ± 1.140^{c}	< 0.001
Tertiary	11.80 ± 3.834^{a}	5.4 ± 1.673^{b}	2.8 ± 0.836^{b}	< 0.001
Atretic	14.80 ± 4.494^{a}	24.00 ± 2.915^{b}	26.80 ± 4.438^{b}	0.001

The data is expressed as the mean ± SE and 95% confidence interval for mean. A value of P below 0.05 may be deemed to indicate a statistically significant alteration. The same letters denoted the resemblance of the groups in the same line, while the separate letters reflected the divergence.

Immunohistochemical analysis

Table 2 displays the AMH immunoreactivity produced by granulosa cells in primary, preantral, and secondary follicle types. In both groups treated with MTH, AMH immunoreactivity decreased in these types of follicles, and the decrease was statistically significant between the 20 mg/kg group and the other groups ($p<0.05$). Additionally, there was no statistically significant difference in the follicle types exhibiting AMH immunoreactivity between the 10 mg/kg group and the control group ($p>0.05$). The study also detected that the effects of MTH worsened with an increase in dosage (Figure 2).

Table 2. Immunohistochemical analyses for AMH levels in ovarian tissue of each experimental groups.

Groups	Control	10 mg/kg MTH	20 mg/kg MTH	p
Primary follicules	131.6 (123.2-141.0)[a]	113.8 (123.4-137.7)[a]	109.1 (99.90-120.9)[b]	< 0.001
Preantral follicles	135.5 ± 10.17[a]	130.0 ± 9.103[a]	103.9 ± 10.69[b]	< 0.001
Secondary follicles	120.7 ± 17.22[a]	126.4 ± 20.16[a]	104.2 ± 7.819[b]	< 0.001

The data for the primary follicles is presented as the median (quartile 1-quartile 3), the data for the preantral and secondary follicles is presented as the mean ± SE and 95% confidence interval for mean. A value of P below 0.05 may be deemed to indicate a statistically significant alteration. The same letters denoted the resemblance of the groups in the same line, while the separate letters reflected the divergence.

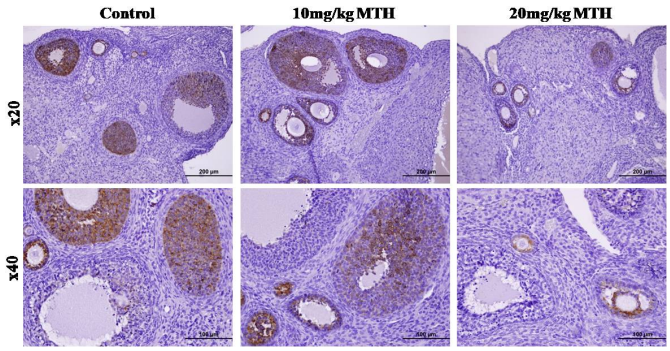

Figure 2. Immnohistochemically stained AMH images in rat ovarian sections from each experimental group. The immunostaining for AMH was particularly apparent in the cytoplasm of the developing granulosa cells. x20 magnification 200x, x40 magnification 400x.

Discussion

MTH has been used for the treatment of a number of conditions, but its use has been limited because of its side effects [18]. MTH has been demonstrated to have adverse effects not only on cancerous cells but also on highly proliferative cells such as ovarian follicles, bone marrow, and gastrointestinal mucosa. In addition, MTH has been demonstrated to exhibit toxicity in a number of organs due to its restricted therapeutic index [19]. This study evaluated the histopathological and immunohistochemical effects of methotrexate on ovarian tissue damage in an experimental animal model. The results showed that two different doses of MTH caused a deterioration of ovarian histology, with a dose-dependent decrease in ovarian follicle reserve observed through both follicle count and AMH immunoreactivity results.

MTH is a medication that is employed in the treatment of various diseases, with varying doses and durations of administration. Because of these differences, its effects on ovarian tissue are also variable. The histopathological examination of rat ovarian tissue following the administration of 20 mg/kg MTH revealed that the growing follicles within the cortex displayed an abundance of fluid-filled spaces and exhibited a reduced number of growing follicles. There were areas of vascular dilatation, congestion, hemorrhage, polymorphonuclear cell infiltration, and intense edema in follicular and luteal cells [20]. Administration of 15 mg/kg MTH every other day for 7 days resulted in oocyte degeneration, separation between granulosa cells of follicles, degenerations in zona pellucida structure, mononuclear cell infiltrations, hemorrhagic areas, especially in the corpus luteum, and vascular congestion [21]. In a study conducted on rats, 1 mg/kg of MTH was administered on days 1, 3, 5, and 7, with plasma AMH levels determined 24 hours after each administration of MTH. The administration of multiple doses of methotrexate did not result in a statistically significant alteration in AMH levels [3]. In a study where MTH was administered as a single dose and multiple doses to patients diagnosed with ectopic pregnancy, follicle stimulation hormone (FSH) and estradiol plasma levels were measured twice before MTH administration and then eight weeks after treatment (3 days), and AMH levels were measured. The number of antral follicles was also evaluated via transvaginal ultrasound before and after the initiation and conclusion of the treatment regimen. The results of the data analysis indicated that there was no significant impact of ovarian reserve and antral follicle count on the efficacy of single-dose and multiple-dose MTH treatment [22]. The histopathological findings were consistent with those of previous experimental studies [8,14], and it was concluded that the adverse effects of MTH in the ovary increased with increasing dose. However, it was also reported that single-dose and multiple-dose MTH treated women diagnosed with ectopic pregnancy exhibited no significant effects on ovarian reserve and antral follicle count.

Researchers have paid little attention to ovarian fibrosis, which is marked by excessive fibroblast proliferation and extracellular matrix deposition, despite its importance for optimal ovarian function. A number of factors, including ovarian disease, surgery, inflammation, and immune abnormalities, can trigger fibrosis in the ovary. If left untreated, this can cause a functional decline of the ovary and increase the risk of infertility in patients [23]. Hortu et al. (2020) reported that 21 days after 20 mg/kg of MTH, there was an increase in the TGF-β level and the percentage of ovarian fibrosis [24]. In this study, MT staining was employed to elucidate the modifications in connective tissue composition and collagen fibers. The 10 mg/kg MTH group exhibited comparable collagen fiber content to the control group. However, the medulla of the 20 mg/kg MTH group exhibited an increase in collagen fibers.

Due to its antimetabolic and cytotoxic effects, the use of MTH is becoming increasingly important in childhood and reproductive age, particularly with regards to its effects on ovarian follicle reserve and fertility [7]. The administration of MTH affected all stages of follicular development. However, the 20 mg/kg group had a marked reduction in the number of primordial, primary, and pre-antral follicles, suggesting that these types of follicles may be more

sensitive to MTH. Although tests such as FSH, inhibin B, and estradiol are used to determine ovarian reserve, the preferred method for obtaining reliable information about the reserve is through the determination of AMH levels [9,25,26]. The plasma level of AMH is strongly correlated with the ovarian follicle reserve and remains unaffected by changes in the ovarian-menstrual cycle [9,27]. A reduction in the AMH level produced by granulosa cells of preantral and antral follicles is observed when these are damaged [28]. The findings of a recent study indicated a reduction in plasma AMH levels in subjects treated with 20 mg/kg MTH, with the decline observed 21 days after the initial treatment [24]. In another study, four weeks after the beginning of the treatment, multiple doses of MTH (1 mg/kg) were administered on days 1, 3, 5, and 7, resulting in a notable decline in AMH levels. In the same study, a reduction in the total number of follicles was considered in parallel with a decline in the AMH level [8]. As documented in the existing literature, the immunohistochemical analysis of AMH immunoreactivity revealed a notable reduction in the 20 mg/kg MTH group when compared to the other groups. The AMH levels and number of ovarian follicles were less influenced in the 10 mg/kg MTH group than in the 20 mg/kg MTH group.

Conclusion

In conclusion, particularly high doses of MTH caused deterioration in the histological and fibrotic structure of the ovarian tissue, together with a reduction in follicular reserve and AMH levels in the developmental stages. In view of these adverse effects of high doses of MTH on the ovaries, it is recommended that the dose of MTH to be administered be determined with great care.

Ethical approval

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee on 05.04.2023 (Decision no: 23/081).

References

1. Khan ZA, Tripathi R, Mishra B. Methotrexate: a detailed review on drug delivery and clinical aspects. Expert Opin Drug Deliv. 2012; 9: 151-169.
2. Karri S, Vanithakumari G. Lipid Alterations Instigated by Methotrexate and Leucovorin in Female Reproductive Tract of Albino Rats. International Journal of Cancer Research and Prevention. 2011; 4(3): 253.
3. Benian A, Guralp O, Uzun DD, et al. The effect of repeated administration of methotrexate (Mtx) on rat ovary: measurement of serum antimullerian hormone (AMH) levels. Gynecol Endocrinol. 2013; 29: 226-9.
4. Furst DE. The rational use of methotrexate in rheumatoid arthritis and other rheumatic diseases. Br J Rheumatol. 1997; 36: 1196-204.
5. Iwase A, Nakamura T, Nakahara T, Goto M, Kikkawa F. Anti-Mullerian hormone and assessment of ovarian toxic treatment: a systematic narrative review. Reprod Sci. 2015; 22: 519-526.
6. Shea B, Swinden MV, Ghogomu ET, Ortiz Z, Katchamart W, Rader T, Bombardier C, Wells GA, Tugwell P. Folic acid and folinic acid for reducing side effects in patients receiving methotrexate for rheumatoid arthritis. Cochrane Database Syst. Rev. 2013;2013(5):CD000951.
7. Gol M, Saygili U, Koyuncuoglu M, et al. Influence of high-dose methotrexate therapy on the primordial follicles of the mouse ovary. J Obstet Gynaecol Res. 2009; 35: 429-433.
8. Soylu Karapinar O, Pinar N, Özcan O, et al. Protective effect of alpha-lipoic acid in methotrexate-induced ovarian oxidative injury and decreased ovarian reserve in rats. Gynecol Endocrinol 2017; 33: 653-659.
9. Taylor HS, Pal L, Seli E. Speroff's clinical gynecologic endocrinology and infertility. In: Taylor HS, Pal L, Seli E (eds), Amenorrhea, 9th edn. Wolters Kluwer, New York, 2020;821-939.
10. Almeida F, Costermans NGJ, Soede NM, Bunschoten A, Keijer J, Kemp B, et al. Presence of anti-Mullerian hormone (AMH) during follicular development in the porcine ovary. PLoS ONE. 2018;13: e0197894.
11. Moolhuijsen LME, Visser JA. Anti-Mullerian hormone and ovarian reserve: Update on assessing ovarian function. J Clin Endocrinol Metab. 2020; 105: 3361-73.
12. Sakalli Çetin E, Tetiker H, İlhan Çelik Ö, Yılmaz N, Ciğerci İH. Methotrexate-Induced Nephrotoxicity in Rats: Protective Effect of Mistletoe (Viscum album L.) Extract. Complement Med Res. 2017;24(6):364-370.
13. El-Agawy MSE-d, Badawy AMM, Rabei MR, Elshaer MMA, El Nashar EM, Alghamdi MA, Alshehri MA, Elsayed HRH. Methotrexate-Induced Alteration of Renal Aquaporins 1 and 2, Oxidative Stress and Tubular Apoptosis Can Be Attenuated by Omega-3 Fatty Acids Supplementation. International Journal of Molecular Sciences. 2022;23(21):12794.
14. Turkler C, Onat T, Yildirim E, Kaplan S, Yazici GN, Mammadov R, Sunar M. An experimental study on the use of lycopene to prevent infertility due to acute oxidative ovarian damage caused by a single high dose of methotrexate. Adv Clin Exp Med. 2020;29(1): 5-11.
15. Onder GO, Balcioglu E, Baran M, Ceyhan A, Cengiz O, Suna PA, Yıldız OG, Yay A. The different doses of radiation therapy-induced damage to the ovarian environment in rats. Int J Radiat Biol. 2021; 97(3): 367-375.
16. Goktepe O, Balcioglu E, Baran M et al. Protective effects of melatonin on female rat ovary treated with nonylphenol. Biotech Histochem. 2022; 98(1):13-19.
17. Onder GO, Goktepe O, Karaman E, Karakas E, Mat OC, Bolat D, Okur E, Tan FC, Balcioglu E, Baran M, Ermis M, Yay A. Nonylphenol Exposure-Induced Oocyte Quality Deterioration Could be Reversed by Boric Acid Supplementation in Rats. Biol Trace Elem Res. 2023;201(9):4518-4529.
18. Asvadi I, Hajipour B, Asvadi A, et al. Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. Eur Rev Med Pharmacol Sci. 2011;15: 1003-9.
19. Padmanabhan S, Triphathi DN, Vikram A, Ramarao P, Jena GB. Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice: intervention of folic and folinic acid. Mutat Res. 2009; 673:43-52.
20. Kiremitli T, Kiremitli S, Akselim B, et al. Protective effect of Coenzyme Q10 on oxidative ovarian and uterine damage induced by methotrexate in rats. Human & Experimental Toxicology. 2021;40(9):1537-1544.
21. Kısmet Aka K, Aydın Acar Ç, Özgöçmen M, Aslankoç R, Yeşilot Ş. Resveratrol allevıates methotrexate- ınduced ovarıan ınjury vıa suppressıng oxıdatıve stress and apoptosıs ın rats. Med J Sdu. 2022;29(3):476-84.
22. Zargar M, ShahAli S, Moramezi F, et al. Effect of Single and Multiple Methotrexate Doses Treatment on Ovarian Reserve in Ectopic Pregnancy Women: A Prospective Cohort Study. J Obstet Gynecol India. 2024.
23. Zhou F, Shi LB, Zhang SY. Ovarian fibrosis: A phenomenon of concern. Chin Med J. 2017; 130: 365-371.
24. Hortu I., Ozceltik G, Ergenoglu AM, et al. Protective effect of oxytocin on a methotrexate-induced ovarian toxicity model. Arch Gynecol Obstet. 2020;301, 1317-1324.
25. Roudebush WE, Kivens WJ, Mattke JM. Biomarkers of ovarian reserve. Biomark Insights. 2008 ;3: 259-268.
26. Ozzola G. Anti-Mullerian hormone: A brief review of the literature. Clin Ter. 2017;168: e14-e22.
27. Liu CM, Ding LJ, Li JY, Dai JW, Sun HX. Advances in the study of ovarian dysfunction with aging. Yi Chuan 2019;41: 816-26.
28. Dewailly D, Andersen CY, Balen A et al. The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update. 2014;20: 370-385.