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Original Article

Histopathological and Immunohistochemical Evaluation of Methotrexate-Induced Gonadal Damage in Rats: Role of SCF, mTOR, and SIRT-1

Sıçanlarda Metotreksata Bağlı Gonadal Hasarın Histopatolojik ve İmmünohistokimyasal Olarak Değerlendirilmesi: SCF, mTOR ve SIRT-1'in Rolü

Kübra Tuğçe KALKAN[1], Betül YALÇIN[2], Özge CENGİZ MAT[3], Arzu Hanım YAY[3]

[1]Kırşehir Ahi Evran University Faculty of Medicine, Department of Histology and Embriyology, Kırşehir, Türkiye
[2]Adıyaman University Faculty of Medicine, Histology and Embriyology, Adıyaman, Türkiye
[3]Erciyes University Faculty of Medicine, Histology and Embriyology, Kayseri, Türkiye

ABSTRACT

Objective: Methotrexate (MTX) is a highly effective chemotherapy for cancer. This drug has a gonadotoxic effect, mainly in the testes and ovaries. Our study used histopathological and immunohistochemical methods to assess the potential damage to testicular and ovarian tissue caused by MTX use.

Methods: Twenty-four Wistar albino rats, both male and female, were used in our study. Four sets of rats; control male, MTX male, control female, and MTX female were created. The male and female MTX-treated groups received a single intraperitoneal dose of 20 mg/kg MTX. The testes and ovaries of rats sacrificed under general anesthesia were extracted and histopathologically analyzed. In addition, the immunoreactivity intensities of stem cell factor (SCF), mechanistic target of rapamycin (mTOR), and SIRT-1 in both tissues were measured by immunohistochemistry.

Results: Johnsen's testicular biopsy score in the testicular seminiferous tubules was significantly lower in the MTX group than in the control group ($p<0.001$). The ovary showed substantial follicular degeneration ($p<0.05$), vascular congestion ($p<0.01$), and fibrosis ($p<0.001$). MTX reduced SCF immunoreactivity density in the testis and ovary ($p<0.05$). Furthermore, MTX reduced mTOR, a marker of autophagy, in the testis ($p<0.05$) and ovary ($p<0.001$) compared with the control. SIRT-1 intensity increased dramatically in the testis ($p<0.001$) and ovary ($p<0.01$) in the injured group, unlike the mTOR marker.

Conclusions: Our investigation revealed that the gonads incurred significant damage as a result of MTX. One vital option for reducing or eliminating this damage to the ovaries and testicles is the use of anti-oxidant-rich substances.

Keywords: Methotrexate, chemotherapy, testis, ovary

ÖZ

Amaç: Metotreksat (MTX) kanser olgularının önde gelen kemotörapatiklerinden biridir. Bu ilaç özellikle testis ve ovaryum üzerinde gonadotoksik bir etkiye sahiptir. Çalışmamızın amacı MTX kullanımına bağlı testis ve ovaryum dokusunda oluşabilecek olası hasarı histopatolojik ve immünohistokimyasal analizlerle araştırmaktır.

Yöntemler: Çalışmamız için 24 adet Wistar albino erkek ve dişi sıçanlar kullanıldı. Bu sıçanlar 4 farklı gruba ayrıldı. Bu gruplar; kontrol erkek, MTX erkek, kontrol dişi ve MTX dişi olarak isimlendirildi. MTX uygulanan erkek ve dişi grubuna 20 mg/kg MTX, tek doz ve intraperitoneal olarak uygulandı. Genel anestezi altında sakrifiye edilen sıçanların testis ve ovaryumları alınarak histopatolojik analizler için kullanıldı. Ayrıca her iki dokuda da kök hücre faktörü (SCF), rapamisinin mekanistik hedefi (mTOR) ve SIRT-1 immünoreaktivite yoğunluğu immünohistokimya ile değerlendirildi.

Bulgular: MTX grubunda testis seminifer tübülünde analizlenen Johnsen testis biyopsisi skoru kontrol grubuna kıyasla istatistiksel anlamda azalış gösterdi ($p<0,001$). Ovaryumda ise MTX tedavisi kontrol grubuna nazaran gözle görülür bir hasar meydana getirdi. Bu grupta foliküler dejenerasyon ($p<0,05$), damar konjesyonu ($p<0,01$) ve fibrozis ($p<0,001$) belirlendi. Hem testis hem de ovaryumda SCF immünoreaktivite yoğunluğu MTX grubunda azalma gösterdi ($p<0,05$). Ayrıca otofaji ile ilişkili belirteçlerden mTOR kontrol grubuna nazaran MTX gruplarında testis ($p<0,05$) ve ovaryumda ($p<0,001$) anlamlı bir şekilde azaldı. SIRT-1 yoğunluğu ise mTOR belirtecinin aksine hasar grubuda testis ($p<0,001$) ve ovaryumda ($p<0,01$) anlamlı bir artış gösterdi.

Sonuçlar: Sonuç olarak, araştırmamızda MTX'in testis ve yumurtalık üzerindeki olası olumsuz etkilerini değerlendirmek adına histopatolojik ve immünohistokimyasal analizler gerçekleştirdik. Ve analizlerimiz MTX tedavisinin gonadlar üzerinde kayda değer bir hasar oluşturduğunu bize gösterdi. Testis ve yumurtalık üzerindeki bu hasarın azaltılması veya tamamen ortadan kaldırılması adına antioksidan içeriklerinin kullanımı oldukça önemli bir alternatif olacaktır.

Anahtar kelimeler: Metotreksat, kemoterapi, testis, ovaryum

Address for Correspondence: K.T. Kalkan, Kırşehir Ahi Evran University Faculty of Medicine, Department of Histology and Embriyology, Kırşehir, Türkiye
E-mail: tugce.kalkan@ahievran.edu.tr **ORCID ID:** orcid.org/0000-0001-7461-277X

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INTRODUCTION

Cancer is a growing global health concern, with an estimated 18.1 million new cases and 10 million cancer-related deaths worldwide in 2020[1,2]. One of the most popular methods for treating tumors is the use of cancer-fighting drugs or chemotherapy. Many of these medications have negative effects and may permanently damage several tissues in the body and organs[3,4]. Chemotherapy does not only target cancer cells but also affects healthy cells, causing gonadotoxicity, hepatotoxicity, cardiotoxicity, nephrotoxicity, neurotoxicity, and hematological system damage[1,2]. Methotrexate (MTX), a key chemotherapeutic treatment for many kinds of cancer, is a folate inhibitor that swiftly attacks dividing cells. Numerous autoimmune conditions, including multiple sclerosis, scleroderma, rheumatoid arthritis, psoriasis, Crohn's disease, and systemic lupus erythematosus, are also dealt with effectively with MTX[5]. The pathophysiology of MTX-induced organ and tissue damage involves elevated levels of reactive oxygen species, oxidative stress, and inflammatory processes[6]. Testicular damage that could result in infertility is the most dangerous possible adverse effect of MTX[7]. MTX may alter the testicular microenvironment, reducing the number of spermatogonial stem cells essential for sperm production[8]. Prior studies have linked the use of MTX to damage the testicular seminiferous tubules, a fall in sperm count, sperm DNA damage, and decreased spermatogenesis[9]. Many chemotherapy medications, including MTX, which is highly effective in curing rapidly dividing neoplastic cells, have significant side effects on female gonads. Unfortunately, MTX has long-term effects on ovarian function. It causes amenorrhea and possibly menopause. The gonadotoxic effect of this drug is believed to cause infertility, especially in young patients, by reducing the number of ovarian follicles[10]. MTX, which is additionally frequently employed in ectopic and molar pregnancy treatments, boosts the amount of free oxygen radicals and pro-inflammatory compounds[6]. SCF is released by Sertoli cells in the testes, which stimulates germ cell growth. It stimulates oocyte development and is released by granulosa cells in the ovary. Testicular germ cells and mature oocytes in the ovary express high c-kit receptor expression[11]. A well-known serine/threonine kinase protein, mechanistic target of rapamycin (mTOR) is specifically responsible for energy homeostasis, metabolism, protein synthesis, and cellular growth. Under specific circumstances, such as stress, low energy, or oxidative damage, mTOR is suppressed and autophagy is activated[12]. SIRT-1 is a crucial autophagy regulator that promotes autophagy, particularly in stressful or energy-deficient situations. It maintains cell growth and autophagy in control of mTOR. In the case of a lack of energy, SIRT-1 is active and initiates autophagy by deacetylating autophagy-initiating proteins, whereas mTOR is inhibited[13]. Although chemotherapy remains an effective treatment for various cancers, its adverse effects on the reproductive system are well documented. The link between SCF, mTOR, and SIRT-1 in the effects of MTX on the testes and ovaries has not received significant attention in the literature. The current research aimed to fill in information gaps regarding the influence of vital signaling pathways, such as SCF, mTOR, and SIRT-1, on the detrimental effects of MTX on reproductive organs. The present study aimed to investigate the histopathological and immunohistochemical effects of MTX toxicity on testes and ovary tissue in rats.

The Animals

Erciyes University Animal Experiments Local Ethics Committee Animal Experiments Local Ethics Committee approved our experimental guidelines (decision no: 24/057, date: 06.03.2024). Wistar albino adult male and female rats (n=24), aged 8-10 weeks, weighed 150-250 g, and were housed in standard accommodation under stress-free conditions (21°C; 12-12 cycles of light and dark). Animal experiments were performed according to the Animal Research: Reporting *In Vivo* Experiments guidelines[14].

Study Design and Experimental Groups

The Wistar rats were randomly assigned to 4 groups: Control male (n=6), MTX male (MTX Male; n=6), control female (n=6), and MTX female (MTX Female; n=6). In the experiment, male and female Wistar rats not exposed to MTX were used as the control group. On the first day of the experiment, a single intraperitoneal dose of 20 mg/kg MTX (Koçak Farma, Türkiye) was administered to male and female rats receiving the treatment[15]. One day following the initial treatment, all animals were dissected under general anesthesia with xylazine (10 mg/kg body weight intraperitoneally) and ketamine (60 mg ketamine hydrochloride/kg body weight intraperitoneally). The testis and ovary tissue were taken for histopathologic and immunohistochemistry analyses.

Histopathological Evaluation

At the end of the experiment, tissue samples were collected and fixed in 10% formaldehyde. The testes and ovary tissue were then rinsed under tap water and placed through a series of increasing grades of alcohol. Blocks were formed by embedding them in paraffin after clearing with xylol. Hematoxylin&Eosin (H&E) and Masson's trichrome (MT) were used to stain 5-µm sections, which were then passed through an increasing alcohol series,

xylol, and a coverslip before being evaluated under a light microscope [Olympus BX51 (Olympus Corp., Tokyo, Japan)]. Testicular tissue stained with hematoxylin and eosin was analyzed and evaluated using standard light microscopy, scoring it according to Johnsen's criteria[16]. Five locations within each of the 10 seminiferous tubules in each section were sampled to calculate Johnsen's testicular biopsy score (JBTS). For each tubule, JBTS was estimated based on the total number of cells and maturation (Table 1). In addition, ovarian tissue damage was evaluated in terms of follicular degeneration, vascular congestion[17], and fibrosis[18]. The assessment was graded semi-quantitatively on a scale of 0 to 3 (0: None, 1: Mild, 2: Moderate, 3: Severe) for each criterion.

Immunohistochemistry

SCF, mTOR, and SIRT-1 expression in testis and ovary tissue was demonstrated using immunohistochemistry. Immunohistochemical staining was performed using the avidin-biotin peroxidase assay. 5 µm sections from paraffin blocks were prepared on polylysine slides for staining. Sections were deparaffinized in xylol, maintained in each descending alcohol, and finally stored in distilled water to dehydrate them. Sections were boiled in a microwave oven at 600 W with 5% citrate buffer for antigen retrieval, washed with phosphate-buffered saline (PBS), and treated with 3% H_2O_2 to prevent endogenous peroxidase activity. The immunohistochemistry staining kit (Lab VisionTM UltraVisionTM Large Volume Detection System: Anti-polyvalent, HRP, TA-125-HL) was used in the following steps, and the entire procedure was performed in a chamber that prevented the tissues from drying out. Block serum was applied to PBS-washed sections for 10 min at room temperature to ensure that the regions outside the anti-genic areas were covered. Subsequently, sections of mTOR (Cell Signaling Technology 7C10-1:100), SIRT-1 (NBP1 51641- 1:500), and SCF (Santa Cruz Biotechnology-1:450) primary anti-bodies were incubated at 4°C overnight. The sections were then incubated with biotinylated secondary anti-bodies. After washing PBS, streptavidin-peroxidase complex was used. In the next step diaminobenzidine (Diaminobenzidine chromogen and substrate system, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to highlight the immunoreactivity. To enhance nuclear staining, Gill's hematoxylin was used as a counterstain on the sections. Under a light microscope, ovarian sections stained with immunohistochemistry were analyzed, and microscopic images of 10 randomly chosen areas were obtained. The immunoreactivity intensities of the markers identified in the photographs were assessed using ImageJ software. The ImageJ software's color threshold plugin was used to assess all immunoreactivity[19,20].

Statistical Analysis

Version 9 of Graph Pad Prism was used for all statistical analyses. The Shapiro-Wilk test was used to determine data distribution. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) and the Kruskal-Wallis test were used. The Bonferroni test for one-way ANOVA and the Dunn test for the Kruskal-Wallis analysis both revealed significant post hoc comparisons of the variables. A p-value of less than 0.05 was considered statistically significant for all data.

RESULTS

Histopathological Results of the Testicular and Ovarian Tissues

The histomorphological evaluation of the testicular tissues of the control group revealed a normal structure in the cells of the spermatogenic series located on the basement membrane and seminiferous tubule outline structures. The number of spermatogenic series cells in the seminiferous tubules decreased and tubular vacuolization increased in the testicular tissues of the rats in the experimental group that administered MTX. Furthermore, the asymmetric seminiferous tubule

Table 1. Johnsen's scoring system was used for qualitative assessments of fertility and spermatogenesis.

Scores	Histological findings	Score	Histological findings
1	There are no visible germs or Sertoli cells. Atrophic tubules have been identified.	6	A few round spermatids were observed.
2	Only Sertoli cells are present; germ cells are absent.	7	Despite the absence of sperm, many rounds of spermatids were observed.
3	There are no primary spermatocytes. Germ cells were just as spermatogonium primary spermatocytes.	8	The sperm count is very low.
4	A few primordial spermatocytes were identified.	9	Although there are many sperm, they are not round, and the lumen lacks a regular form.
5	There are no and round spermatids. Too many primary spermatocytes were observed.	10	There are many sperm with consistently rounded edges in the lumen, indicating full spermatogenesis.

morphology and tubular architecture resulting in necrosis were noted. Additionally, each time we examined the testicular tissue stained with H&E-evaluated JBTS, we found that the MTX group had a considerably lower score than the control group (p<0.001). The interstitial connective tissue structure in the testicular tissues of the control group was within healthy limits after MT staining was used to identify possible variations in connective tissue. In the MTX-treated group, the interstitial connective tissue area of the testicular tissues did not change in this respect (Figure 1) (Table 2A). We examined the ovarian tissues of both control and MTX-treated mice under a light microscope. Various stages of follicle development were observed in the cortical layer of the ovary in the control group. The surrounding granulosa appeared healthy, and the oocytes were located near the center. There were many blood arteries and loose connective tissue in the medulla. Normal vascular structures were observed. The group that received MTX treatment had unfavorable ovarian tissue. In this group, degenerative follicular structures were identified (p<0.05) (Figure 2 and Figure 3A). Furthermore, the vascular congestion structures were appealing (p<0.01) (Figure 2 and Figure 3B). Analyses of connective tissue formation in ovarian tissues were performed by examining sections stained with MT stain. There was an apparent increase in collagen in the medulla layer in the MTX group (p<0.001) (Figure 2 and Figure 3C) (Table 2B).

SCF Immunoreactivity of Testicular and Ovarian Tissue

When testicular tissue was examined for SCF immunoreactivity intensity, a high reactivity was found in the spermatogonium of the spermatogenic series cells of the seminiferous tubule in the control group. SCF immunoreactivity in the testicular tissues of the MTX-treated group dropped statistically substantially (p<0.05). We examined the intensity of SCF immunoreactivity in

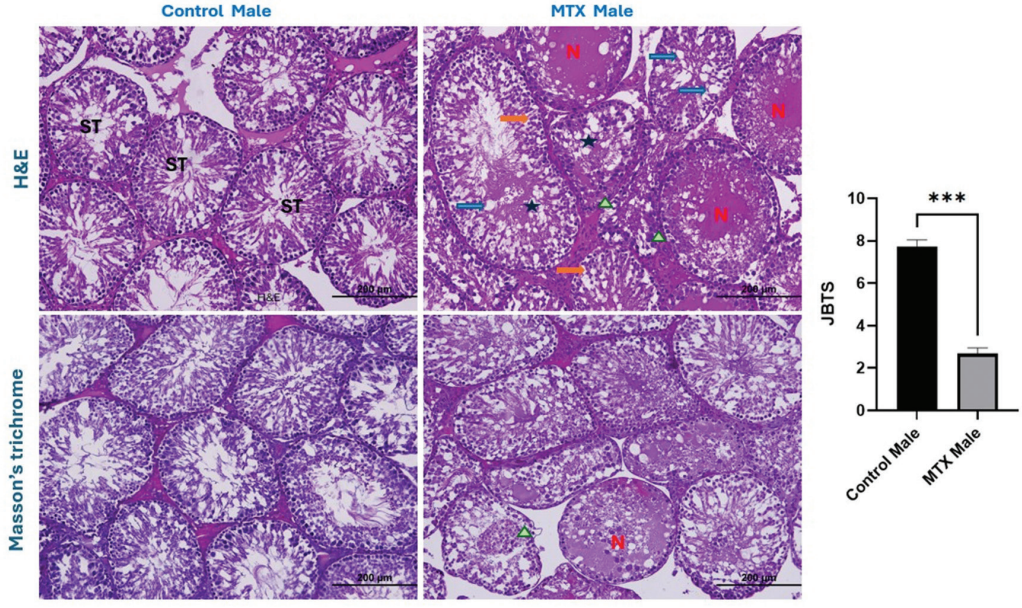

Figure 1. Light microscopic findings and JBTS of rat testis tissue. Control male group; ST: Seminiferous tubules. MTX Male group; N: Necrotic seminiferous tubules; orange arrow: descending spermatogenic lineage cells; blue arrow: tubular vacuolization; green triangle: Asymmetrical seminiferous tubule morphology. H&E: hematoxylin-eosin and MT staining (Olympus BX51, Tokyo, Japan. X20). JBTS graph of the experimental groups. Data are presented as mean ± standard deviation or median (min-max). *: p<0.05, **: p<0.01, ***: p<0.001

JBTS: Johnsen's testicular biopsy score, MTX: Methotrexate

Table 2A. Testis JBTS statistics.

Testis histoscore	Control male	MTX male	p-value
JBTS	8,000 (4,000-10.00)	3,000 (1,000-6,000)	<0,001

The Mann-Whitney U test was used to evaluate non-normal data distribution. Med. (min-max): The quartile value range is shown inside the lines of brackets, whereas the median value is outside.

JBTS; Johnsen's testicular biopsy score, Med: Median, MTX: Methotrexate

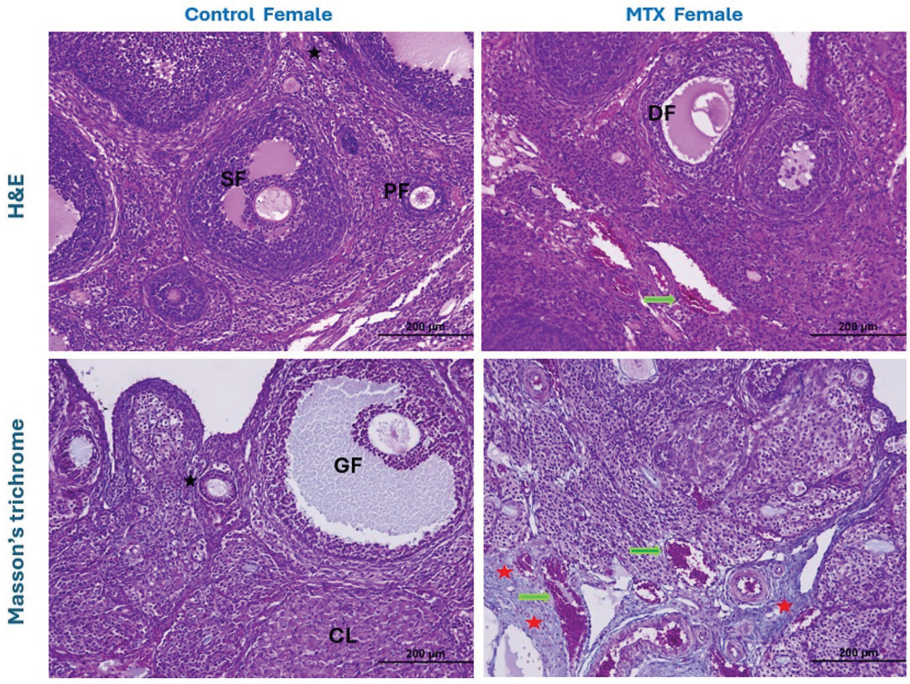

Figure 2. Light microscopic findings of the rat ovary tissue. Control Female group: Several developmental phases of folliculi are observed. Primordial follicles (*), primary follicles (PF), secondary follicles (SF), and Corpora lutea (CL) with large, weakly pigmented acidophilic cells, mature Graafian follicles (GF). MTX Female group: Degenerative follicles (DF). Green arrow, vascular congestion; red star, fibrosis. H&E: hematoxylin-eosin and Masson's trichrome staining (Olympus BX51, Tokyo, Japan. X20).

MTX: Methotrexate, H&E: Hematoxylin&Eosin.

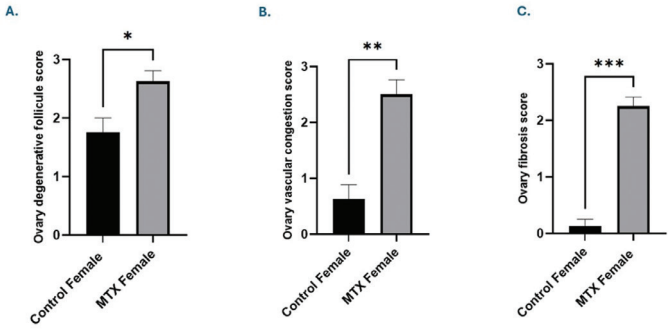

Figure 3. Histopathological findings of rat ovary tissue. Graph exhibiting degenerative follicles, vascular congestion, and fibrosis in the experimental groups. Data are presented as mean±standard deviation or median (min-max). *: p<0.05, **: p<0.01, ***: p<0.001.

MTX: Methotrexate, Min-max: Minimum-maximum.

ovary tissue and found high expression levels in follicles at various stages of development, the corpus luteum, and in ovary stromal cells. In the ovarian tissue of the MTX-treated group, SCF expression was extremely low. Comparing this group with the control group, there was a statistically significant decrease in immunoreactivity (p<0.05) (Figure 4 and Table 3).

mTOR Immunoreactivity of Testicular and Ovarian Tissue

mTOR is expressed mostly in seminiferous tubule cells, interstitial connective tissue, and vessel walls in this region. Conversely, the group that received MTX treatment in testes showed diminished immunoreactivity intensity in the same area (p<0.05). When mTOR expression was measured in ovarian tissue, positivity was high in the corpus luteum and stroma of the control group. The granulosa layer of the follicles exhibited very little reactivity. In the MTX treatment group, mTOR immunoreactivity was considerably lower than that in the control group (p<0.001) (Figure 5 and Table 3).

SIRT-1 Immunoreactivity of Testicular and Ovarian Tissue

The SIRT-1 immunoreactivity intensity of testicular and ovarian tissue was also examined. The primary spermatocytes in the seminiferous tubules exhibit a very poor reaction in testicular tissue of the control group. All spermatogenic series cells, including primary spermatocytes, showed a significant increase in immunoreactivity intensity in response to MTX injury in the testicular tissue of the MTX-treated group, except

Table 2B. Ovary histoscore statistics.			
Ovary histoscore	**Control female**	**MTX female**	**p-value**
Degenerative follicle	2,000(1,000-3,000)	3,000(2,000-3,000)	0.039
Vascular congestion	0,5000(0.000-2,000)	3,000(1,000-3,000)	0.002
Fibrosis	0,000(0.000-1,000)	2,000(2,000-3,000)	<0.001

The Mann-Whitney U test was used to evaluate non-normal data distribution Med.(min-max): The quartile value range is shown inside the lines of brackets, whereas the median value is outside.

Med: Median, Min-max: Minimum-maximum, MTX: Methotrexate.

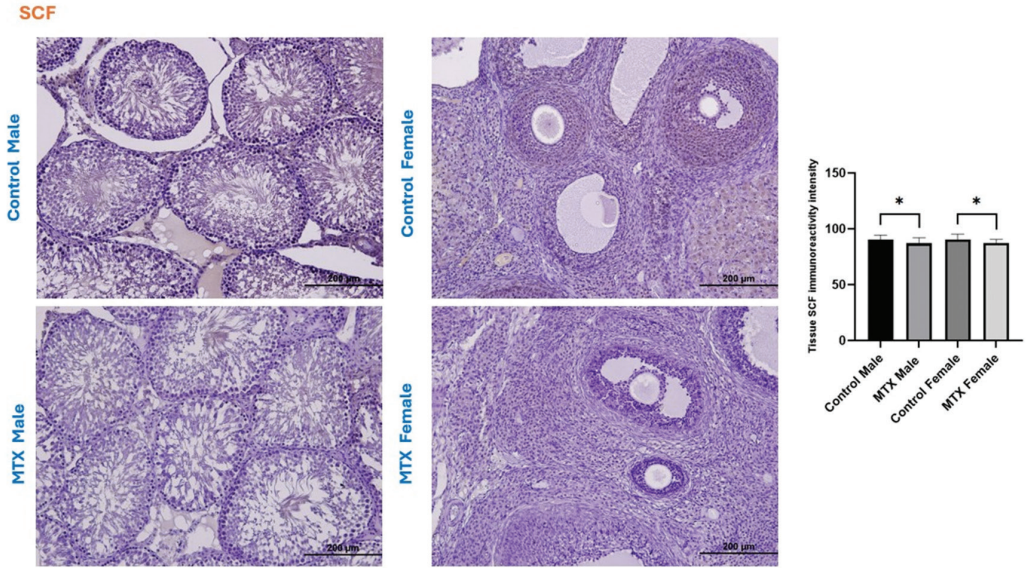

Figure 4. Immunohistochemical microscopic findings and graphs of SCF markers in testicular and ovarian tissues from the experimental groups. The brown areas indicate immunostaining. The slides were counterstained with hematoxylin. Data are presented as mean±standard deviation or median (min-max). *: p<0.05, **: p<0.01, ***: p<0.001. (Olympus BX51, Tokyo, Japan. X20).

SCF: Stem cell factor, Min-max: Minimum-maximum, MTX: Methotrexate.

for spermatogonium (p<0.001). In the granulosa cells of the follicles in the ovarian tissue of the control group, SIRT-1 was negative. The granulosa cells of the follicles, corpus luteum, and ovarian stroma, however, showed exceptionally high immunoreactivity intensity of this marker in the MTX group (p<0.01) (Figure 6 and Table 3).

DISCUSSION

MTX is an anti-metabolite with an extensive list of uses is MTX, which is used to treat autoimmune disorders and cancer. It is frequently selected to treat a wide range of conditions, especially because of the features that affect cell division and DNA synthesis. However, this effective medication may target the reproductive system. MTX's harmful effects of MTX on testicular and ovarian tissues can result in serious medical challenges because these tissues are composed of rapidly dividing cells[5]. Oxidative stress is a known adverse effect of MTX. Its activation in the testes can lead to a rise in free radical generation, impairing spermatogenesis and ultimately resulting in infertility[7]. According to Felemban et al.[21] MTX led to structural disruption and an atypical layout of the spermatogenesis cycle, resulting in the shedding of germ cells into the tubular lumen, severe degeneration in most seminiferous tubules, and significant decreases in Leydig cells and sperm. In this study, we found that MTX administration led to an erosion in the seminiferous tubule morphology and a decline in the spermatogenic series in the testicular tissue seminiferous tubules. Consistent with our findings, another MTX study that assessed testicular injury observed a substantial reduction in the JBTS score of the MTX-treated group compared with the control group[22]. In addition to all the previous studies, our findings suggest that the substantially decreased JBTS scores in the MTX-treated group indicate severe damage to the seminiferous tubules and serious degradation of the spermatogenesis process. The reduced number of

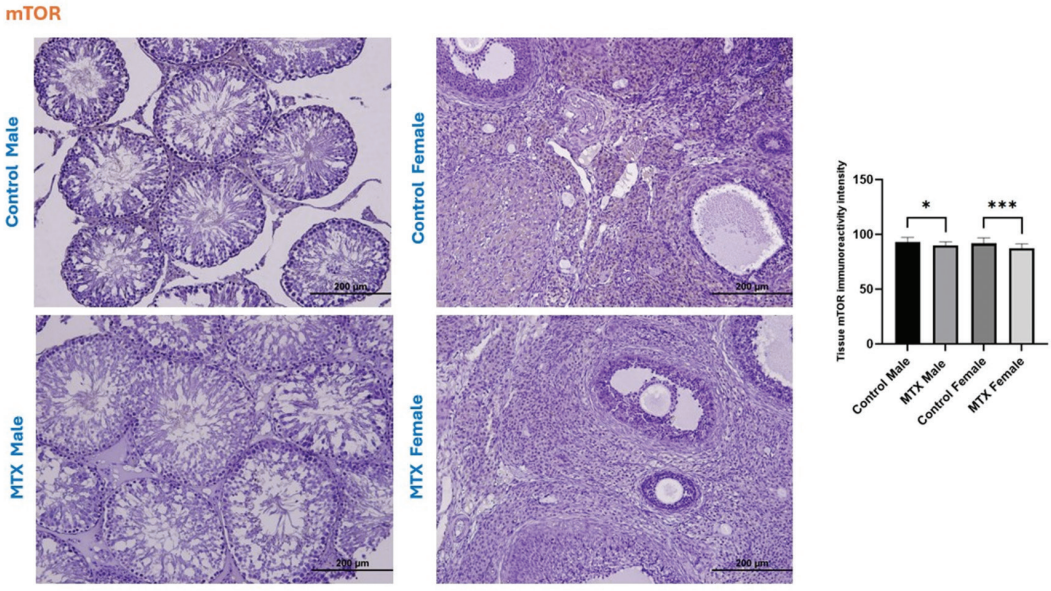

Figure 5. Immunohistochemical microscopic findings and graphs of mTOR markers in testicular and ovarian tissues from the experimental groups. The brown areas indicate immunostaining. The slides were counterstained with hematoxylin. Data are presented as mean±standard deviation or median (min-max). *: p<0.05, **: p<0.01, ***: p<0.001. (Olympus BX51, Tokyo, Japan. X20).

MTX: Methotrexate, mTOR: Mechanistic target of rapamycin, Min-max: Minimum-maximum.

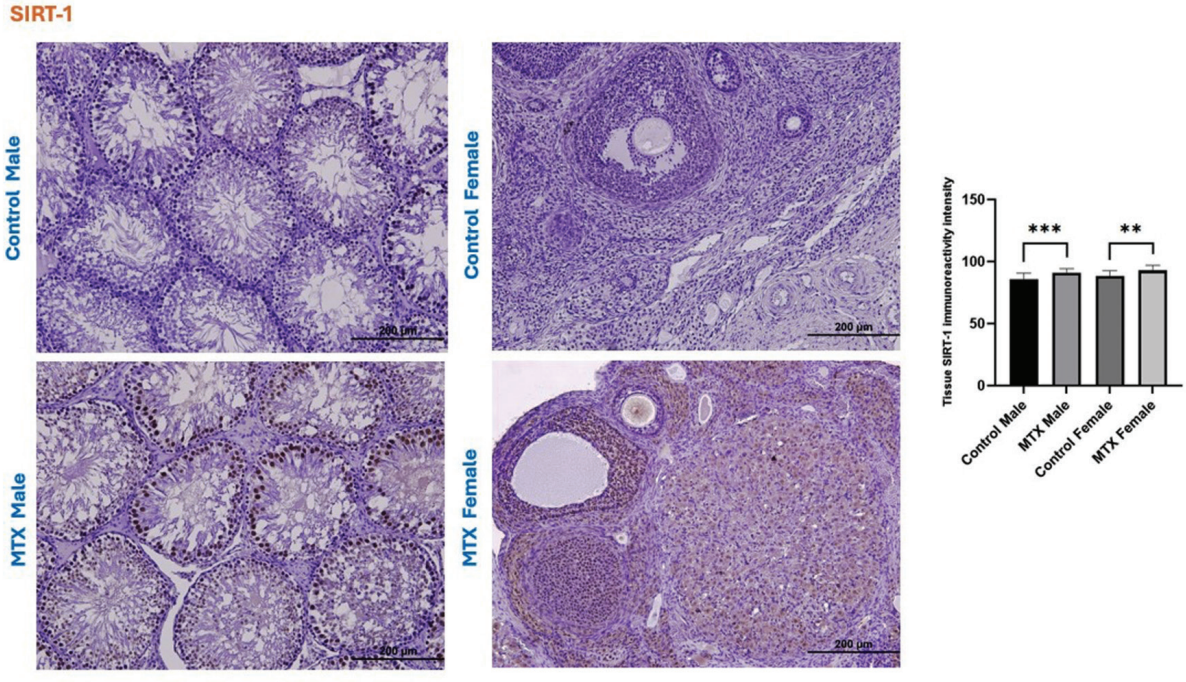

Figure 6. Immunohistochemical microscopic findings and graphs of SIRT-1 markers in testicular and ovarian tissues from the experimental groups. The brown areas indicate immunostaining. The slides were counterstained with hematoxylin. Data are presented as mean±standard deviation or median (min-max). *: p<0.05, **: p<0.01, ***: p<0.001. (Olympus BX51, Tokyo, Japan. X20).

Min-max: Minimum-maximum, MTX: Methotrexate.

Table 3. Statistics of immunohistochemistry findings.					
IHC marker	**Control male**	**MTX male**	**Control female**	**MTX female**	**p-value**
SCF	91.06 (82.19-96.86)	88.34 (73.45-96.71)	90.23 (79.29-98.59)	88.06 (80.49-92.31)	<0.001
mTOR	93.09±4.065	89.93±3.293	91.83± 5.036	87.45±4.036	<0.001
SIRT-1	87.44(70.41-93.35)	90.84 (84.79-98.17)	87.82 (81.51-97.81)	93.29 (81.51-99.59)	<0.001
IHC: Immunohistochemistry, SCF: Stem cell factor, mTOR: Mechanistic target of rapamycin, MTX: Methotrexate.					

spermatogonial stem cells and boosted vacuolization support the gonadotoxic effects of MTX in testicular tissue. Ovarian toxicity associated with MTX includes a reduction in the number of antral follicles, a decrease in anti-Müllerian hormone (AMH) levels, and ultimately a decrease in ovarian reserve[23]. Kiremitli et al.[24] analyzed ovarian tissue following mtx-induced oxidative stress and revealed that the number of degenerative follicles and vascular congestion increased in the MTX group. In another study, MTX therapy in the ovary resulted in a substantial increase in fibrosis compared with the control group. This investigation confirmed our findings regarding MTX dosage and fibrosis[25]. Increased follicular degeneration indicates that MTX causes significant stress and oxidative damage to cells that support oocyte formation. Increased vascular congestion and fibrosis imply that this process involves inflammatory components. These findings imply that MTX has a detrimental effect on ovarian reserve and can increase the risk of infertility in women. SCF, or Kit ligand, is the c-kit ligand for the tyrosine protein kinase receptor. SCF is mostly found in Sertoli cells in testes and is expressed exclusively in germ cells during development[26]. In the fully developed ovary, c-kit is mostly expressed in oocytes, whereas granulosa cells release SCF, a well-known oocyte development booster[27]. In a recent study, it was discovered that the amount of SCF protein decreased considerably in the MTX group compared with the control group[28]. In the latest ovarian study, SCF immunoreactivity intensity was lower in the damaged group than in the control group[29]. This research concluded that SCF immunoreactivity decreased in both testicular and ovarian tissue in the MTX treatment groups, which confirms previous findings. Sperm and oocyte damage results from MTX-induced inhibition of the folate pathway, which also affects DNA synthesis and cell proliferation. Stem cell factor (SCF) is essential for both the regeneration of damaged tissue and the defense of germ cells against such damage. If SCF production is elevated during MTX-induced cellular damage, germ cell regeneration can be enhanced. Autophagy is a well-known self-cannibalization system that adapts to changes in intracellular and extracellular environments during biosynthesis. On the other hand, unusual autophagy can be produced by specific atypical stimuli and stresses, leading to damage[30]. SIRT-1 is a type of sirtuin found in mammals. Sirtuins are nicotinamide adenine dinucleotide-dependent deacetylase that regulate cellular metabolism, proliferation, and genome stability. It affects various cellular processes, including cancer, aging, metabolic regulation, and differentiation. Additionally, it regulates autophagy by initiating the process[31]. The mTOR kinase is a well-known Ser/Thr protein kinase with a molecular weight of 290 kDa that regulates the cell energy status and metabolism[32]. mTOR is a crucial regulator of autophagy. SIRT-1 deficiency leads to enhanced mTOR signaling[33]. SIRT-1 expression was investigated in a hamster study in which bisphenol caused testicular injury, and it was discovered that this marker was diminished in the damaged group[34]. In another testicular study, contrary to our findings, SIRT-1 expression in radiation-induced testis decreased significantly[35]. The results of these studies were reflected by a decrease in the level of this indicator in contrast with a increase in SIRT-1 immunoreactivity intensity in our MTX-treated testicular tissue. In an experiment where mTOR expression was assessed, it emerged that as the formaldehyde dose climbed in testicular tissue, mTOR immunopositivity was lessened compared with that in the control groups[36]. In a rat study in which lead provoked testicular toxicity, mTOR levels were lower in the lead group than in the control group, which is consistent with our results. Despite the toxicity of lead, the anti-oxidant component used in the study increased the level of this protein to levels approximately comparable to those of the control group[37]. Several studies have revealed that SIRT contributes to ovarian aging[38-40]. Transgenic mice with ovarian SIRT1 overexpression had lower mTOR levels and a higher ovarian survival rate[41]. In an ovarian examination, increasing amounts of Ti_3C_2 nanosheets progressively aggravated ovarian tissue damage, resulting in a steady decline in mTOR activity[42]. Onder et al.[29] analyzed the SIRT/mTOR pathway in non-ylphenol-induced ovarian damage. This study determined that SIRT immunoreactivity intensity increased while mTOR immunoreactivity intensity decreased in the NP-exposed group compared with the control group, which is in line with our results. All studies and our data indicate that MTX induces stress reactions in cells, which triggers

the autophagy system. Testicular and ovarian cells activate autophagy in response to MTX therapy. In this mechanism, mTOR inhibition and SIRT-1 activation disrupt spermatogenesis and oocyte development, resulting in germ cell loss and impaired reproductive function. This study confirmed that MTX has gonadotoxic effects on both the testes and ovaries. Impaired spermatogenesis in males and follicular degeneration in females suggest that MTX triggers infertility in young patients. Autophagy-regulating therapies should be investigated to minimize MTX-induced cellular stress and damage. Therapeutic techniques that specifically modify SIRT-1 may be beneficial for reproductive organ maintenance.

CONCLUSION

As a result, in our research, we evaluated the effects of MTX on the testicles and ovaries and performed histopathological and immunohistochemical analyses. This study demonstrated that MTX at a given dose caused significant damage to both testicular and ovarian tissues. In addition to histological evidence in the gonads, a decrease in SCF in the MTX treatment group, as well as immunoreactivity of SIRT-1 and mTOR expressions, which are autophagic pathways that develop by promoting another process, indicate damage. Unfortunately, the negative effects of chemotherapy on the reproductive system are visible. To minimize testicular and ovarian damage, a variety of anti-oxidant components are used. Our findings suggest that anti-oxidant therapies can be employed to lessen and prevent MTX's detrimental impact on the reproductive system. Potent anti-oxidants may be valuable to developing approaches that protect reproductive health, and they will probably be utilized in research examining their potential for reducing oxidative damage and cellular stress caused by MTX. Additionally, these anti-oxidants, along with SCF, may assist in managing autophagy processes through the SIRT-1 and mTOR signaling pathways.

Ethics

Ethics Committee Approval: Erciyes University Animal Experiments Local Ethics Committee Animal Experiments Local Ethics Committee approved our experimental guidelines (decision no: 24/057, date: 06.03.2024).

Informed Consent: Since this study was conducted on animals, patient consent was not required.

Footnotes

Author Contributions

Surgical and Medical Practices: B.Y., Ö.C.M., Concept: K.T.K., A.H.Y., Design: K.T.K., A.H.Y., Data Collection and/or Processing: B.Y., Ö.C.M., Analysis and/or Interpretation: K.T.K., A.H.Y., Literature Search: K.T.K., Writing: K.T.K.

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REFERENCES

1. Kızıl HE, Caglayan C, Darendelioğlu E, et al. Morin ameliorates methotrexate-induced hepatotoxicity via targeting Nrf2/HO-1 and Bax/Bcl2/Caspase-3 signaling pathways. Mol Biol Rep. 2023;50:3479-88.
2. Varışlı B, Caglayan C, Kandemir FM, Gür C, Bayav İ, Genç A. The impact of Nrf2/HO-1, caspase-3/Bax/Bcl2 and ATF6/IRE1/PERK/GRP78 signaling pathways in the ameliorative effects of morin against methotrexate-induced testicular toxicity in rats. Mol Biol Rep. 2022;49:9641-9.
3. Gunyeli I, Saygin M, Ozmen O. Methotrexate-induced toxic effects and the ameliorating effects of astaxanthin on genitourinary tissues in a female rat model. Arch Gynecol Obstet. 2021;304:985-97.
4. Wei T, Wang L, Tang J, Ashaolu TJ, Olatunji OJ. Protective effect of Juglanin against doxorubicin-induced cognitive impairment in rats: Effect on oxidative, inflammatory and apoptotic machineries. Metab Brain Dis. 2022;37:1185-95.
5. Hamed KM, Dighriri IM, Baomar AF, et al. Overview of Methotrexate Toxicity: A Comprehensive Literature Review. Cureus. 2022;14:e29518.
6. Yucel Y, Oguz E, Kocarslan S, et al. The effects of lycopene on methotrexate-induced liver injury in rats. Bratisl Lek Listy. 2017;118:212-6.
7. Sarman E, Gulle K, Ilhan I. Histochemical, Immunohistochemical, and Biochemical Investigation of the Effect of Resveratrol on Testicular Damage Caused by Methotrexate (MTX). Reprod Sci. 2023;30:3315-24.
8. Delkhosh A, Shabani F, Delashoub M. Experimental accelerating testicular tissue recovery post-methotrexate treatment in rats: A promising role of Sertoli cell-conditioned medium: An experimental study. Int J Reprod Biomed. 2024;22:295-304.
9. Soliman A, Alkafafy M, Hassan FW, Zakaria M, Abduljabbar MH, Mohamed K, et al. Investigating the Protective Role of L-carnitine and Thymoquinone against Methotrexate-Induced Testicular Damage in Rats. Pak Vet J. 2024;44:314-321.
10. Ata N, Kulhan NG, Kulhan M, et al. The effect of resveratrol on oxidative ovary-damage induced by methotrexate in rats (Resveratrol oxidative ovary-damage). Gen Physiol Biophys. 2019;38:519-24.
11. Foster BM, Langsten KL, Mansour A, Shi L, Kerr BA. Tissue distribution of stem cell factor in adults. Exp Mol Pathol. 2021;122:104678.
12. Mizushima N, Levine B. Autophagy in Human Diseases. N Engl J Med. 2020;383:1564-76.
13. Lyu Y, Meng Z, Hu Y, et al. Mechanisms of mitophagy and oxidative stress in cerebral ischemia-reperfusion, vascular dementia, and Alzheimer's disease. Front Mol Neurosci. 2024;17:1394932.

14. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010;8:e1000412.

15. Radwan SM, Alqulaly M, Elsaeed MY, Elshora SZ, Atwa AH, Wasfey EF. L-carnitine reverses methotrexate-induced nephrotoxicity in experimental rat model: Insight on SIRT1/PGC-1α/Nrf2/HO-1 axis. J Appl Toxicol. 2023;43:1667-75.

16. Johnsen SG. Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones. 1970;1:2-25.

17. Ayazoglu Demir E, Mentese A, Kucuk H, Turkmen Alemdar N, Demir S. p-Coumaric acid alleviates cisplatin-induced ovarian toxicity in rats. J Obstet Gynaecol Res. 2022;48:411-9.

18. Atilgan R, Pala Ş, Kuloğlu T. Investigation of treatment efficacy of 10% povidone-iodine sclerotherapy on ovarian cyst diameter: an experimental study. Turkish journal of medical sciences. 2019;49:795-801.

19. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat Rec (Hoboken). 2013;296:378-81.

20. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9:676-82.

21. Felemban SG, Aldubayan MA, Alhowail AH, Almami IS. Vitamin B17 Ameliorates Methotrexate-Induced Reproductive Toxicity, Oxidative Stress, and Testicular Injury in Male Rats. Oxid Med Cell Longev. 2020;2020:4372719.

22. Yalcin A, Aydin H, Turk A, Dogukan M, Eser N, Onderci M, et al. Vitamin D: An effective way to combat methotrexate-induced testis injury. Medicine. 2020;9:998-1003.

23. Anğın AD, Çöğendez E, Gün İ, et al. The Effect of Dehydroepiandrosterone on Ovarian Reserve in Ovarian Damage Caused by Methotrexate. Medical Laboratory Technology Journal. 2020;6-1.

24. Kiremitli T, Kiremitli S, Akselim B, et al. Protective effect of Coenzyme Q10 on oxidative ovarian and uterine damage induced by methotrexate in rats. Hum Exp Toxicol. 2021;40:1537-44.

25. Hortu I, Ozceltik G, Ergenoglu AM, Yigitturk G, Atasoy O, Erbas O. Protective effect of oxytocin on a methotrexate-induced ovarian toxicity model. Arch Gynecol Obstet. 2020;301:1317-24.

26. Cardoso HJ, Figueira MI, Socorro S. The stem cell factor (SCF)/c-KIT signalling in testis and prostate cancer. J Cell Commun Signal. 2017;11:297-307.

27. Figueira MI, Cardoso HJ, Correia S, Maia CJ, Socorro S. Hormonal regulation of c-KIT receptor and its ligand: implications for human infertility? Prog Histochem Cytochem. 2014;49:1-19.

28. Mohammed SS, Mansour MF, Salem NA. Therapeutic Effect of Stem Cells on Male Infertility in a Rat Model: Histological, Molecular, Biochemical, and Functional Study. Stem Cells Int. 2021;2021:8450721.

29. Onder GO, Goktepe O, Karaman E, et al. Nonylphenol Exposure-Induced Oocyte Quality Deterioration Could be Reversed by Boric Acid Supplementation in Rats. Biol Trace Elem Res. 2023;201:4518-29.

30. Xue J, Gruber F, Tschachler E, Zhao Y. Crosstalk between oxidative stress, autophagy and apoptosis in hemoporfin photodynamic therapy treated human umbilical vein endothelial cells. Photodiagnosis Photodyn Ther. 2021;33:102137.

31. Wu QJ, Zhang TN, Chen HH, et al. The sirtuin family in health and disease. Signal Transduct Target Ther. 2022;7:402.

32. Moreira BP, Oliveira PF, Alves MG. Molecular Mechanisms Controlled by mTOR in Male Reproductive System. Int J Mol Sci. 2019;20:1633.

33. Tatone C, Di Emidio G, Barbonetti A, et al. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. Hum Reprod Update. 2018;24:267-89.

34. Kumar J, Verma R, Haldar C. Melatonin ameliorates Bisphenol S induced testicular damages by modulating Nrf-2/HO-1 and SIRT-1/FOXO-1 expressions. Environ Toxicol. 2021;36:396-407.

35. El-Mesallamy HO, Gawish RA, Sallam AM, Fahmy HA, Nada AS. Ferulic acid protects against radiation-induced testicular damage in male rats: impact on SIRT1 and PARP1. Environ Sci Pollut Res Int. 2018;25:6218-27.

36. Fang J, Li DH, Yu XQ, et al. Formaldehyde exposure inhibits the expression of mammalian target of rapamycin in rat testis. Toxicol Ind Health. 2016;32:1882-90.

37. Elhemiely AA, Yahia R, Gad AM. Naringenin alleviate reproductive toxicity evoked by lead acetate via attenuation of sperm profile and biochemical alterations in male Wistar rat: Involvement of TGFβ/AKT/mTOR pathway. Journal of Biochemical and Molecular Toxicology. 2023;37:e23335.

38. Long GY, Yang JY, Xu JJ, et al. SIRT1 knock-in mice preserve ovarian reserve resembling caloric restriction. Gene. 2019;686:194-202.

39. Vo KCT, Sato Y, Kawamura K. Improvement of oocyte quality through the SIRT signaling pathway. Reprod Med Biol. 2023;22:e12510.

40. Yan F, Zhao Q, Li Y, et al. The role of oxidative stress in ovarian aging: a review. J Ovarian Res. 2022;15:100.

41. Zhang J, Fang L, Lu Z, et al. Are sirtuins markers of ovarian aging? Gene. 2016;575:680-6.

42. Yang L, He Z, Hu L, et al. Ti3C2 nanosheet-induced autophagy derails ovarian functions. Journal of Nanobiotechnology. 2024;22:242.