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Evaluation of the Effects of Acute Cisplatin Administration on Testicular and Ovarian Tissue in Rats

Sıçanlarda Akut Sisplatin Uygulamasının Testis ve Yumurtalık Dokusundaki Etkilerinin Değerlendirilmesi

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ABSTRACT

Aim: Cisplatin, one of the effective chemotherapeutics in cancer treatment, has the potential to affect the testis and ovary, leading to permanent or temporary infertility. This study aimed to determine the acute effects of cisplatin on testis and ovary histology and to investigate whether it induces any changes in immunohistochemical cyclooxygenase-2 (COX2), nuclear factor kappa B p65 (NFκB-p65), and heat-shock protein 70 (HSP70) levels.
Material and Methods: The study was planned as four groups: male control, male cisplatin, female control, and female cisplatin. The cisplatin group rats were adminestered with 7 mg/kg cisplatin, and all rats were sacrificed 24 hours later. Hematoxylin-eosin and Masson's trichrome stains were applied to testis and ovary tissues to examine their histopathological structure, and an immunohistochemistry staining protocol was applied to determine immunostaining intensity of COX2, NFκB-p65, and HSP70.
Results: In the cisplatin group, a decrease in seminiferous tubule epithelium, an elevation in fibrotic response in the interstitial area, and a notable reduction in Johnson testicular biopsy score ($p<0.001$) were seen in the testis. In the ovary, atretic follicles ($p=0.006$) and luteal structures within the cortex, as well as vascular congestion ($p=0.001$), edema ($p=0.001$), and fibrotic areas within the medulla, were evident. These alterations resulted in a statistically significant increase in ovarian histoscores, except for leukocyte infiltration ($p=0.322$). In both tissues, cisplatin significantly increased the immunostaining intensity of COX2, NFκB-p65, and HSP70 compared to the control group.
Conclusion: Acute cisplatin administration can induce tissue damage and pro-inflammatory response in the testis and ovary.
Keywords: Cisplatin; testis; ovary; cyclooxygenase-2; NF-kappa B p65; heat-shock protein 70.

ÖZ

Amaç: Kanser tedavisinde etkili kemoterapötiklerden biri olan sisplatin, testis ve overi etkileyerek kalıcı veya geçici infertiliteye yol açma potansiyeline sahiptir. Bu çalışma, sisplatinin testis ve over histolojisi üzerindeki akut etkilerini belirlemeyi ve immünohistokimyasal siklooksijenaz-2 (COX2), nükleer faktör kappa B p65 (NFκB-p65) ve ısı-şok protein 70 (HSP70) düzeylerinde herhangi bir değişikliğe neden olup olmadığını araştırmayı amaçlamıştır.
Gereç ve Yöntemler: Çalışma dört grup olarak planlandı: erkek kontrol, erkek sisplatin, dişi kontrol ve dişi sisplatin. Sisplatin grubu sıçanlara 7 mg/kg sisplatin uygulandı ve tüm sıçanlar 24 saat sonra sakrifiye edildi. Testis ve over dokularına histopatolojik yapılarını incelemek amacıyla hematoksilen-eozin ve Masson trikrom boyaları uygulandı ve COX2, NFκB-p65 ve HSP70'in immün boyama yoğunluğunu belirlemek amacıyla immünohistokimya boyama protokolü uygulandı.
Bulgular: Sisplatin grubunda, testiste seminifer tübül epitelinde azalma, interstisyel alanda fibrotik yanıtta artış ve Johnson testiküler biyopsi skorunda kayda değer bir azalma ($p<0,001$) görüldü. Overde, kortekste atretik foliküller ($p=0,006$) ve luteal yapılar yanı sıra medullada vasküler konjesyon ($p=0,001$), ödem ($p=0,001$) ve fibrotik alanlar belirgindi. Bu değişiklikler, lökosit infiltrasyonu ($p=0,322$) dışında, over histoskorlamasında istatistiksel olarak anlamlı bir artışla sonuçlandı. Her iki dokuda da, sisplatin, kontrol grubuna kıyasla COX2, NFκB-p65 ve HSP70 immün boyanma yoğunluğunu önemli ölçüde artırdı.
Sonuç: Akut sisplatin uygulaması, testiste ve overde doku hasarına ve proinflamatuar yanıta yol açabilir.
Anahtar kelimeler: Sisplatin; testis; over; siklooksijenaz-2; NF-kappa B p65; ısı-şok protein 70.

INTRODUCTION

Since obtaining Food and Drug Administration approval in 1978, cisplatin has been utilized as the first chemotherapy drug to contain heavy metal compounds due to its platinum content. It has been employed both as a monotherapy and in combination with other antineoplastic agents (1,2). The cytotoxic effects of this drug are mediated by its ability to bind to DNA, leading to cell cycle arrest and DNA damage, which in turn activate apoptosis (3,4). Cisplatin has the potential to impact the physiological functions of various systems by inducing toxicity in both healthy and tumor cells (5). Furthermore, the toxicity of cisplatin, which may have adverse effects on numerous organs, including the reproductive system (6), restricts its use in treating various types of solid tumors. Due to the presence of cells with elevated mitotic activity, the testis and ovary are particularly susceptible to chemotherapeutic agents (7,8).

Heat shock proteins are molecules induced in response to a variety of physiological and environmental stimuli, including inflammatory processes. These molecules, which also function as molecular chaperones, contribute to the protection of cells by recognizing misfolded and mutated proteins (9-11). One of these molecules, heat-shock protein 70 (HSP70), is capable of fulfilling anti-apoptotic, anti-inflammatory, and antioxidant functions based on its intracellular and extracellular localization (12). The HSP70 protein has the capacity to inhibit the activity of nuclear factor kappa B (NFκB), which in turn reduces the expression of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), thus attenuating inflammatory responses in various cell types (13).

The transcription factor NFκB serves a regulatory function in immune responses, stimulating the activity of cyclooxygenase-2 (COX2) and iNOS enzymes and the release of proinflammatory mediators. This molecule has been demonstrated to be sensitive to the effects of oxidative stress (14). Moreover, abnormalities in the NFκB signaling pathway have been linked to a range of pathological disorders, including various inflammatory diseases and malignancies. Accordingly, the inhibition of NFκB may facilitate an effective strategy for the alleviation of ovarian damage. One of the inflammatory agents encoded by the NFκB gene, COX2, has been demonstrated to enhance cytokine production concomitant with reactive oxygen species (ROS) production in normal tissues (15,16).

The objective of this study was to ascertain the impact of cisplatin on the histology of rat testicular and ovarian tissue, as well as on the immunoreactivity of COX2, NFκB p65 (NFκB-p65), and HSP70.

MATERIAL AND METHODS

Animal Care

Approval for the utilization of twelve male and twelve female adult Wistar albino rats sourced from the Erciyes University Experimental Animal Application and Research Centre was obtained from the Animal Experiment Local Ethics Committee of the Erciyes University (dated 06.03.2024, and numbered 24/056). The rats had a 12-hour light/dark cycle, were kept in hygienic cages at 22-24°C, and were fed commercial pellets and water in accordance with their preferences.

Experimental Design

The experiment was composed of four groups, with a total of twenty-four animals, as detailed below: Control male group: The rats were not subjected to any treatment. Cisplatin male group: A single dose (7 mg/kg) of cisplatin was administered intraperitoneally (17). Control female group: The rats were not subjected to any treatment. Cisplatin female group: A single dose of (7 mg/kg) cisplatin was administered intraperitoneally (18).

Twenty-four hours after cisplatin administration, all animals were euthanized using a xylazine (10 mg/kg) and ketamine (60 mg/kg) combination as general anesthetic agents. Testicular and ovarian tissues were promptly subjected to a 10% formaldehyde solution for subsequent histopathological and immunohistochemical examination.

Histopathological Examination

Following a fixation period of 72 hours in formaldehyde, a histological follow-up procedure was conducted. After the completion of the dehydration, transparency, and paraffin blocking processes, respectively, 5 µm-thick sections were obtained. For the purpose of enabling examination under a light microscope, the tissue sections underwent staining with hematoxylin and eosin (H&E) and Masson's trichrome (MT).

The degree of testicular damage was quantified using the Johnsen testicular biopsy score (JTBS). Histological evaluations of seminiferous tubules according to the JTBS and identification were given in Table 1 (19). Accordingly, X indicates normal spermatogenesis, IX indicates multiple spermatozoa with irregularities in the germinal epithelium, VIII indicates a small number of spermatozoa, VII-II indicates maturation arrest, and a score of I indicates the complete absence of cells in the seminiferous tubules (20).

In order to detect ovarian damage, twenty non-overlapping fields were randomly selected for analysis at 40x magnification, and the mean values for each group were subsequently evaluated. Ovarian scoring was conducted in accordance with the criteria for hemorrhage, edema, follicular degeneration, vascular congestion, and leukocyte infiltration. In order to assess the severity of the histopathological damage in the ovarian tissue section, a scoring system that adopted the specified criteria was employed, with scores ranging from 0 to 3 (21-23).

Immunohistochemistry Analysis

The testis and ovary sections on polylysine-coated slides underwent immunohistochemical staining for COX2, NFκB-p65, and HSP70 by the avidin-biotin peroxidase method. The paraffin removal process, utilizing xylene, rehydration with a series of progressively lower alcohol concentrations, and subsequent rinsing with distilled water, was completed in that order. The sections for antigen retrieval were incubated with citrate buffer solution in a 95°C microwave for 10 min, followed by incubation in the same solution and duration at room temperature. After washing in PBS, they were incubated with 3% hydrogen peroxide for 12 minutes to eliminate endogenous peroxidase activation. Following exposure to the ultra V block component of the immunostaining kit, primary antibodies were applied to the sections overnight at 4°C. The biotinylated secondary antibody solution and the peroxidase-conjugated streptavidin solution, both provided in the kit, were incubated for 10 minutes each. Three times, PBS washing was conducted at the conclusion

Table 1. Qualitative histological assessments of the seminiferous tubules according to the Johnsen testicular biopsy score

Score	Identification of histological findings
1	There are no cellular structures within the tubules.
2	Sertoli cells are present in the absence of germ cells.
3	Among the germ cells, only spermatogonia are available.
4	Only a few spermatocytes are present.
5	The presence of several or many spermatocytes in the absence of spermatozoa and spermatids.
6	The presence of a few spermatids in the absence of spermatozoa.
7	The presence of many spermatids in the absence of spermatozoa.
8	Only a few spermatozoa are present.
9	The presence of many spermatozoa and germinal epithelial debris in the tubular lumen.
10	There are numerous spermatozoa resulting from spermatogenesis and tubules with regular germinal epithelium and lumen.

of each chemical treatment. The sections were subjected to chromogen staining with diaminobenzidine and core staining with Mayer hematoxylin. Subsequent to the application of distilled water, a series of progressively higher alcohol concentrations and xylenes were employed to impregnate the sections with entellan. In order to ascertain the degree of immunostaining for COX2, NFκB-p65, and HSP70 in the testis and ovary, ten distinct areas from each rat in the experimental group were evaluated using the ImageJ software program.

Statistical Analysis

The normality of the histopathological scoring and immunohistochemical measurements of the ovarian and testicular tissues was established through the utilization of both the Shapiro-Wilk and Kolmogorov-Smirnov tests. Non-normally distributed immunostaining data were evaluated using the Mann-Whitney U test, and as descriptive statistics, the median with interquartile range, and minimum-maximum were presented. The data analysis, conducted with the aid of the GraphPad Prism 9 program (San Diego, CA), was accepted as statistically significant if the p-value was less than 0.05.

RESULTS

Histopathological Results

In HE-stained sections, the testicular tissue of control group rats was observed to comprise seminiferous tubules and interstitial connective tissue. The seminiferous tubule epithelium displayed a normal testicular architecture, comprising cells of the spermatogenic series at varying developmental stages. These cells were observed to be located from the basement membrane to the lumen, with spermatozoa present in the lumen. In the acute cisplatin-treated rats, a reduction in the number of spermatozoa was observed within the lumen of some tubules, with the absence of spermatozoa noted in others. Furthermore, the seminiferous tubule epithelium was observed to be disorganized, with a tendency to shed into the lumen (Figure 1).

The assessment of MT-stained sections was undertaken with the objective of determining the presence of fibrotic changes in the testicular tissue. The control group testes exhibited no alterations in the capsule thickness or the area of connective tissue within the tubules. In the group treated with cisplatin, an increase in fibrotic tissue was observed, notably in the vessel walls within the connective tissues, when compared to the control group (Figure 1A).

In terms of JTBS score, a notable decrease was discerned in the cisplatin group in comparison to the control group, with a statistical significance ($p<0.001$, Table 2). The JTBS findings, together with the related images representing the histology of the experimental groups, were presented in Figure 1B.

Table 2. Comparison of JTBS between the groups

	Control	Cisplatin	p
JTBS	10 (8-10) [7-10]	5 (4-5) [1-9]	**<0.001**

JTBS: Johnsen testicular biopsy score, descriptive statistics were presented as median (25^{th}-75^{th} percentile) [minimum-maximum]

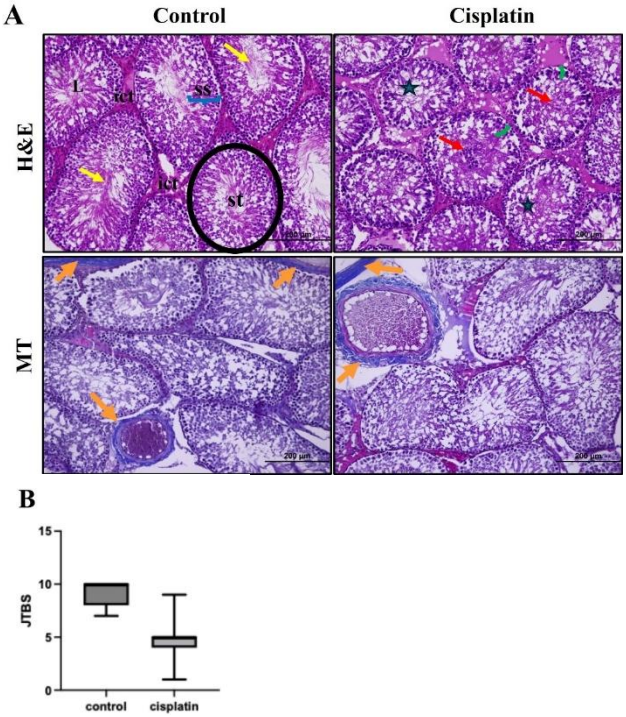

Figure 1. A) HE and MT stained testicular sections, and **B)** JTBS of experimental groups

HE: hematoxylin-eosin staining, 200x, MT: Masson's trichrome staining, 200x, JTBS: Johnsen testicular biopsy score, seminiferous tubules (st, encircled by black circle), lumen (L), interstitial connective tissue (ict), spermatogenic series (ss, blue bracket), sperm (yellow arrow), shedding of spermatogenic cells (red arrow), reduction of the quantity of spermatozoa (star), decreased seminiferous tubule epithelial thickness (green bracket), fibrosis (orange arrow)

In HE-stained sections, the ovarian tissue of the control group appeared healthy, with its medulla and cortex containing follicles at different stages of development. In the cisplatin-treated group, the cortex exhibited a prevalence of corpus luteum and follicles with impaired structure. In this group, the scores of vascular congestion (p=0.001), hemorrhage (p<0.001), edema (p=0.001), and follicular degeneration (p=0.006), except for the leukocyte infiltration (p=0.322), were significantly elevated in comparison to the control group (Figure 2, Table 3).

MT-stained ovarian sections of the cisplatin group exhibited blue-stained fibrotic structures extending from the medulla of the ovarian tissue to the cortex. These fibrotic structures were not present in the control group (Figure 2A).

Immunohistochemical Analysis

After acute cisplatin-induced testicular and ovarian toxicity, immunohistochemical staining was performed on testicular and ovarian sections to determine the immunoreactivity of COX2, NFκB-p65, and HSP70 proteins (Table 4). In the cisplatin groups, there was a notable enhancement in COX2, NFκB-p65, and HSP70 immunostaining intensity in both the testis (p<0.001, p=0.049, p<0.001) and ovary (p=0.045, p=0.005, p=0.010) when compared to the control group (Figures 3 and 4).

DISCUSSION

In the cisplatin-administered group, deterioration in the structure of the epithelial cells of the seminiferous tubules was observed, along with a reduction in the number of spermatozoa. Administration of cisplatin resulted in an increase in the immunoreactivity of COX2, NFκB-p65, and HSP70 in the testicular tissue. Similar to these results, in ovarian tissue, the administration of cisplatin was demonstrated to result in the occurrence of histopathological alterations, including the presence of hemorrhage, edema, follicular degeneration, and vascular congestion. Furthermore, an increase in the immunostaining intensities of both COX2 and NFκB-p65, as well as HSP70, was observed in the ovary of this group. The findings indicated that acute dose cisplatin induced damage to testicular and ovarian tissue, accompanied by a notable increase in COX2, NFκB-p65, and HSP70 immunostaining intensity.

On the 7th day of the 14-day experimental period, the cisplatin administration at a dose of 7 mg/kg/day was observed to induce irregularities in the spermatogenic series, a reduction in germinal cells, and a shrinkage of cells in interstitial areas within the testicular tissue (24). Four days after 7 mg/kg cisplatin administration in the present study resulted in tubule degeneration and a reduction in germinal cells, accompanied by a decrease in

Table 3. Comparison of ovarian scoring results

	Control	Cisplatin	p
Vascular congestion	0 (0-0.5) [0-1]	2 (0.5-2) [0-3]	**0.001**
Hemorrhage	0 (0-0) [0-1]	2 (1-3) [0-3]	**<0.001**
Leukocyte infiltration	0 (0-0) [0-1]	0 (0-1) [0-1]	0.322
Edema	0 (0-0) [0-1]	1 (1-2) [0-2]	**0.001**
Follicular degeneration	0 (0-1) [0-1]	1 (0.5-2) [0-3]	**0.006**

descriptive statistics were presented as median (25th-75th percentile) [minimum-maximum]

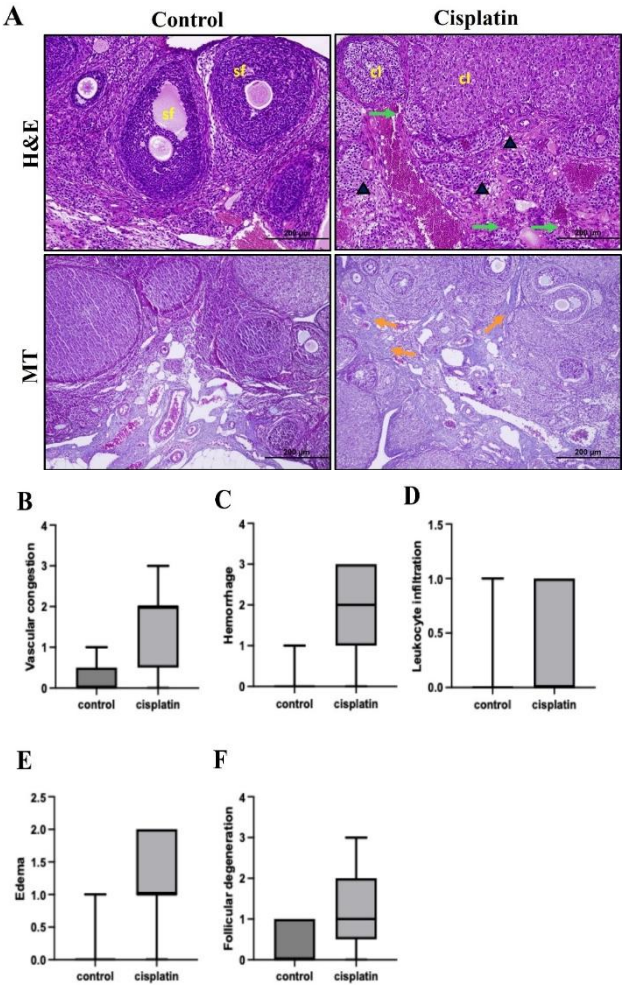

Figure 2. A) HE and MT stained ovarian sections, and **B)** vascular congestion, **C)** hemorrhage, **D)** leukocyte infiltration, **E)** edema, **F)** follicular degeneration scores

HE: hematoxylin-eosin staining, 200x, MT: Masson's trichrome staining, 200x, secondary follicle (sf), corpora lutea (cl), vascular congestion (green arrow), edema (arrowhead), fibrosis (orange arrow)

Table 4. Analysis of immunostaining intensity for COX2, NFκB-p65, and HSP70 in testis and ovarian tissue

		Control	Cisplatin	p
Testis	**COX2**	92.10 (89.56-92.10) [81.15-102.5]	94.39 (90.08-102.7) [84.08-127.4]	**<0.001**
	NFκB-p65	89.92 (86.67-94.61) [73.56-109.1]	91.19 (87.45-97.67) [81.99-148.1]	**0.049**
	HSP70	93.82 (91.01-95.95) [84.87-99.88]	99.27 (94.22-102.9) [84.87-158.5]	**<0.001**
Ovary	**COX2**	90.72 (86.06-94.91) [80.33-99.02]	92.19 (88.79-98.67) [81.20-140.8]	**0.045**
	NFκB-p65	86.11 (83.98-88.69) [75.44-97.86]	89.95 (86.65-93.66) [74.13-98.30]	**0.005**
	HSP70	91.19 (88.59-93.61) [80.62-99.52]	92.88 (90.03-96.28) [85.81-99.87]	**0.010**

COX2: cyclooxygenase-2, NFκB: nuclear factor kappa B, HSP70: heat-shock protein 70, descriptive statistics were presented as median (25th-75th percentile) [minimum-maximum]

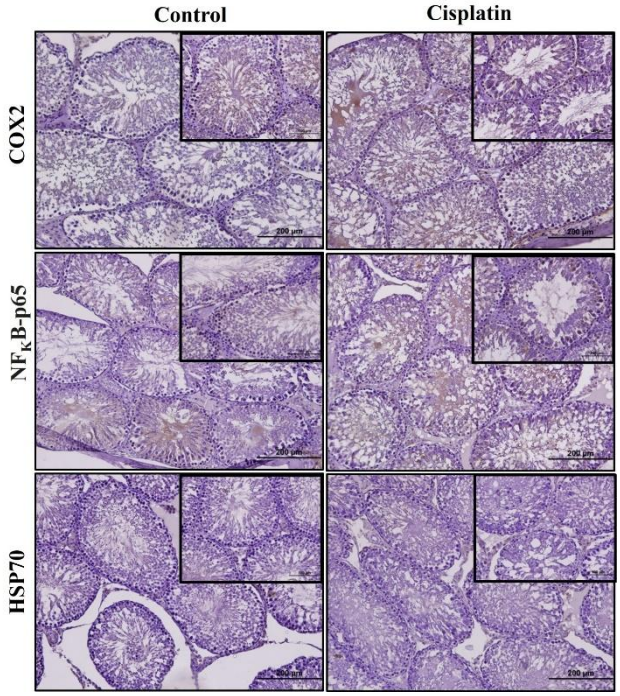

Figure 3. The immunoreactivity of COX2, NFκB-p65, and HSP70 in rat testicular tissue sections
COX2: cyclooxygenase-2, NFκB: nuclear factor kappa B, HSP70: heat-shock protein 70, larger images are x20, 200x, and upper left images are x40, 400x

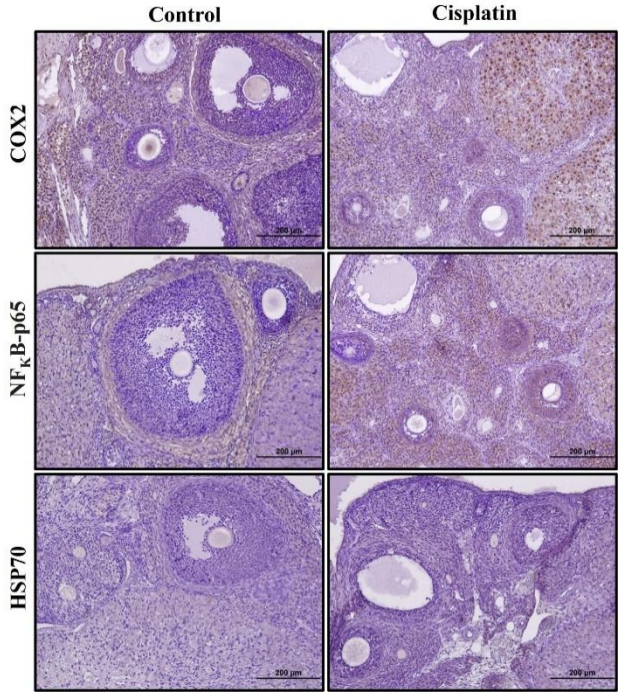

Figure 4. The immunoreactivity of COX2, NFκB-p65, and HSP70 in rat ovarian tissue sections
COX2: cyclooxygenase-2, NFκB: nuclear factor kappa B, HSP70: heat-shock protein 70, x20 magnification 200x

the thickness of the germinal epithelium (25). The histological findings demonstrated the presence of desquamation and disorganization of germinal cells, along with degenerative changes in these cells, thereby corroborating the deterioration in sperm quality in cisplatin-administered rats (24). In MT-stained testis, the presence of capsule thickening was discernible on days 7 and 14, while the emergence of perivascular fibrosis could only be observed on day 14 (26). The ovarian damage induced by cisplatin was comparable to the findings of the study in which 2.5 mg/kg cisplatin was administered once a day for 14 days. However, the severity of the damage was more pronounced in comparison to the 7 mg/kg dose employed in this study. The observed effect was thought to be related to both the increased dosage of cisplatin administered and the longer duration of exposure (21). In an investigation of the role of cisplatin treatment administered on the 21st day of a 28-day experiment on ovarian follicle reserve, a reduction in primary follicles and secondary follicles was observed, accompanied by an increase in damaged follicles and luteal structures (18). Furthermore, it has been reported that the fertility capacity of rats may be diminished as a consequence of cisplatin-induced ovarian morphological degeneration and a reduction in serum anti-müllerian hormone (AMH) levels (27). Seven days following cisplatin administration, an increased deposition of collagen was evident in both the tunica albuginea located beneath the surface epithelium and the stroma encircling the follicles (23). The outcomes of previous research studies corroborated the histopathological alterations associated with cisplatin on both the testis and ovary revealed in the present investigation.

In order to prevent cisplatin-induced reproductive toxicity, it is of great importance to inhibit the NFκB, oxidative stress, and inflammatory pathways (28). The overexpression of the NFκB-p65 protein has been shown to stimulate the expression of proinflammatory cytokines, particularly interleukin-1 beta (IL-1β) and TNF-α. These cytokines have been demonstrated to induce testicular inflammation (29). In the testis, a significant positive immunostaining was observed within the cytoplasm of both interstitial and spermatogenic cells three days following the administration of cisplatin (24). A study demonstrated that the administration of cisplatin on the 7th day of a 14-day experimental period resulted in an increase in TNF-α and NFκB-p65 protein levels in testicular tissue, as evidenced by western blot analysis (20). In the ovary, according to the carried out studies, the levels of the pro-inflammatory cytokines NFκB, IL-1β, IL-6, TNF-α, COX2, and iNOS were all significantly elevated (18,21).

HSP70 is observed to be present in low quantities within the cytoplasm under any conditions (30,11). In the presence of stress, this molecule is increased in the nucleus (30), thereby ensuring the survival of the cell (31). The overproduction of HSP70 was found to suppress the LPS-stimulated augmentation of TNF-α and IL-6 generation by impeding the activation of IκBα and the nuclear translocation of NFκB-p65 in rats (32). Heat shock proteins are crucial for the process of spermatogenesis and for maintaining cellular integrity against external insults, including heat, chemicals, and radiation (33). The expression of HSP70 in the testis of adult rats is influenced by fluctuations in temperature, with elevated levels of protein expression observed in response to temperature changes (34). A notable elevation in HSP70 immunoreactivity was observed in the germinal epithelium and testicular interstitial tissue within experimental injury groups subjected to ecstasy-induced injury. Moreover, it

was reported that there was a significant increase in HSP70 immunoreactivity in spermatogonia, primary spermatocytes, spermatids, Sertoli, and Leydig cells in response to the increased dose of ecstasy compared to the control group (35). The findings of the present study also demonstrated that cisplatin administration led to an increased HSP70 immunostaining intensity in spermatogenic cells and the interstitial tissue. Similarly, under conditions of excessive cellular stress induced by ecstasy, the increased expression of heat shock proteins in spermatogenic cells and the interstitial tissue has been found to exert cytoprotective effects and prevent apoptosis (33,35). Ovarian damage and its severity have been reported to be associated with increased serum HSP70 (36). In cases where premature ovarian failure has been experimentally induced by cyclophosphamide, there has been a notable elevation in the concentration of HSP70, and it is widely accepted that this is related to inflammatory factors (37).

CONCLUSION

The present study provided evidence to support the damage caused by cisplatin in both testicular and ovarian tissue by histopathological evaluations conducted 24 hours after acute cisplatin administration. In testicular and ovarian tissues, an elevation in the immunostaining intensity of COX2, NFκB-p65, and HSP70 was revealed by immunohistochemical analysis. It is recommended that new studies be designed to elucidate the mechanisms underlying the damage, with particular emphasis on the role of the immune response in the reproductive organs following cisplatin administration at varying doses and durations.

Ethics Committee Approval: The study was approved by the Local Ethics Committee on Animal Experiment of Erciyes University (06.03.2024, 24/056).

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