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In vivo Gonadoprotective Effects of Myricetin on Cisplatin-Induced Testicular Damage via Suppression of TLR4/NF-κB Inflammation Pathway and Heat-Shock Response

Efectos Gonadoprotectores *in vivo* de la Miricetina sobre el Daño Testicular Inducido por Cisplatino Mediante la Supresión de la Vía de Inflamación TLR4/NF-κB y la Respuesta al Choque Térmico

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SUMMARY: The aim of this study is to reveal the gonadoprotective effects of myricetin (MYC), which has many biological properties, on cisplatin (CP)-induced testicular damage in rats. For this purpose, 40 male Wistar albino rats were divided into 4 groups as Control (group given no treatment), MYC (group given 5 mg/kg/i.p myricetin for 7 days), CP (group given 7 mg/kg/i.p cisplatin at 7th day) and MYC + CP (group given 5 mg/kg/i.p myricetin for 7 days before 7 mg/kg/i.p cisplatin injection). After administrations, testicular tissues of animals were extracted and processed according to tissue processing protocol. Hematoxylin & Eosin staining were performed to evaluate the histopathological changes and Johnsen'sTesticular Biopsy Score (JTBS) was applied and mean seminiferous tubule diameters (MSTD) were measured to compare experimental groups in terms of histopathological changes. Moreover, TLR4, NF-κB, HSP70 and HSP90 expression levels were detected by immunohistochemical staining and the density of immunoreactivity were measured to determine the difference in the expression levels of these factors among groups. Additionally, testicular apoptosis was detected via TUNEL assay. JTBS and MSTD data were significantly lower in CP group compared to other groups and MYC administrations significantly protects testicular tissue against CP-induced damage. Moreover, TLR4, NF-κB, HSP70 and HSP90 expressions and apoptotic cells significantly increased in the CP group ($p<0.05$). However, MYC administrations exerted a strong gonadoprotective effect on testicular tissue in terms of these parameters in MYC+CP group ($p<0.05$). According to our results, we suggested that MYC can be considered as a protective agent against cisplatin-induced testicular damage.

KEY WORDS. Cisplatin; Heat-shock response; Inflammation; Myricetin; Testicular damage.

INTRODUCTION

As the success rate of chemotherapy has increased, cancer patients' long-term survival rates and quality of life have both improved. Chemotherapeutics, on the other hand, can induce serious damage in healthy organs like the testis (Patel & Kaufmann, 2012). Chemotherapeutics have a deleterious impact on the testis, and chemotherapy-induced testicular toxicity is one of the most well-known and researched adverse effects (Ceylan *et al*., 2020). Cisplatin (CP), CP-diaminedichloroplatin-II, Due to its antiproliferative effects on cancer cells, it is used to treat a number of malignancies, including lung, breast, ovarian, testicular, head, and neck cancers (Dasari & Tchounwou, 2014; Hanif & Hartinger, 2018). However, it has catastrophic consequences for the brain, kidneys, liver, and gonads (Lu & Cederbaum, 2006; Melli *et al*., 2008; Santos *et al*., 2008; Quintanilha *et al*., 2017; Kohsaka *et al*., 2020).

CP-induced gonadotoxicity is an intricate pathophysiological process condition occurring during cancer chemotherapy. Apoptosis, vascular damage, oxidative and endoplasmic reticulum stress, inflammation, and the heat-shock response are some of the intracellular activities it is connected to (Bhat *et al*., 1999; Soni *et al*., 2016; Ceylan *et al*., 2020). Much research has shown that testicular function is impaired in CP-induced gonadotoxicity and, accordingly, various histopathological changes are observed

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in the seminiferous tubules. It has been reported that damage in the seminiferous tubules increased and mean seminiferous tubule diameters substantially decreased in the CP-induced experimental testicular tissue damage models (Ceylan *et al*., 2020).

It is reported that testicular toxicity caused by CP is significantly influenced by the inflammatory response and inflammation-related cytotoxicity (Eid *et al*., 2016; Hamza *et al*., 2016). Inflammation is controlled or regulated by several signaling pathways, each of which is interconnected and impacts the others. The key controller of inflammation, nuclear factor-kappa B (NF-κB), is involved in the etiology of oxidative and inflammatory tissue damage (Liu *et al*., 2017). Transcript-regulating pro-inflammatory cytokine release has demonstrated the impact of NF-κB on tissue damage (Lawrence, 2009). By affecting downstream cytokines and proteins involved in the inflammatory response, NF-κB activation has been suggested to play a major role in testicular injury (Eid *et al*., 2016; Shokri *et al*., 2020). Furthermore, toll-like receptor 4 (TLR4) increases the overexpression of interferon regulatory factor 3 (IRF3), NF-κB, extracellular signal–regulated kinases 1/2 (ERK1/2), and mitogen-activated protein kinases (MAPKs) via an alternate pathway (Yamamoto *et al*., 2003; Kuzmich *et al*., 2017). These several signal pathways work together to increase cytokine release and serious damage increases in the male reproductive system because of this inflammatory response.

Many cells overexpressed numerous stress-related proteins such as heat-shock proteins (HSPs) when they are exposed to stressful circumstances such as thermal stress. Inducible HSPs are defense molecules that are overexpressed in response to numerous processes like inflammation, ischemia, and oxidative stress to keep tissues safe from further damage. The action of HSPs (chaperones) in testicular injury is one of the most effective ways of protecting testicular tissue. Based on their molecular weight, HSPs are split into many subfamilies. HSPs are regularly expressed in healthy testicular tissue, but their levels of expression change when the testis is injured (Akin *et al*., 2022).

Molecular chaperones assist in protein foldings and the assembly of polymeric complexes. Heat shock proteins produce molecular chaperones (HSPs). The molecular weight of HSPs is used to classify them. Members of the HSPs family, one of the largest families of molecular chaperons, include HSP70 and HSP90. They are involved in protein folding and are induced by stress (Craig *et al*., 1993). Furthermore, these two proteins have been linked to disorders such as chronic heat stress (Pei *et al*., 2012), toxicity (Selim *et al*., 2012), cancer (Ren *et al*., 2017), and in experimental models such as the esticular damage model (Öztürk *et al*., 2020).

Many defense mechanisms, such as the immune reaction sent off when cells are injured by illness or hazardous substances, depend strongly on apoptosis (Norbury & Hickson, 2001). Two major apoptotic pathways that have been identified are the intrinsic pathway, also known as the mitochondrial pathway, and the extrinsic pathway, also known as the death receptor pathway. Extrinsic and intrinsic pathways both lead to the execution pathway. This process starts with the activation of Caspase-3, an apoptosis initiator that causes DNA breakage, cytoskeletal and nuclear protein degeneration, apoptotic body formation, and the creation of antigens for phagocytic cell receptors (Martinvalet *et al*., 2005). Several experimental studies have showed that CP chemotherapy induces a serious apoptosis in the testicular tissue (H *et al*., 2020).

We are aware that many different cell types overexpress inflammatory cytokines when an inflammatory response occurs, and that apoptosis is induced via inflammation-related apoptotic pathways. Thus, it is unavoidable that inflammation triggered by CP will not cause overexpression of TLR4 and NF-κB, and heat-shock response induced by CP administrations in the testis tissue will not activate HSPs and cause the overexpression of the HSP70 and HSP70. Therefore, we aimed to demonstrate the potential gonadoprotective effects of MYC on testicular injury caused by CP administrations. For this purpose, histological evaluation of the testicular tissue damage was performed, and CP-induced apoptosis was evaluated. In addition, we compared the expression levels of inflammatory and heat-shock response markers TLR4, NF-κB, HSP70 and HSP90 in the testis. The purpose of this study was to reveal how MYC protects against testicular damage caused by CP.

MATERIAL AND METHOD

Drugs. Myricetin (MYC) was bought from Sigma-Aldrich (San Louis, MO, M6760-25MG). According to the manufacturer, the quality of the powdered MYC was approximately 96 %. For intraperitoneal injections into experimental animals, the powdered MYC was dissolved in a solution of 100 % ethanol and distilled water. Cisplatin (CP) solution was purchased from Koçak Farma (Turkey, 8699828770077) and it was directly injected to experimental animals at proper doses.

Animals. This study was designed and performed according to Directive 2010/63/EU, accepted directive for the

legislation for the protection of animals used for scientific purposes. The Experimental Animal and Local Ethics Committee at Erciyes University approved the study's experimental methodology with number 22/107/2022. Erciyes University's Hakan Cetinsaya Experimental and Clinic Research Center provided 40 male Wistar albino rats (8 weeks old, weighing 200–250 g). The rats were kept in cages, with their nutritional and water demands being fulfilled ad libitum, at a temperature of 21°C and 12 hours of light/dark per day.

Experimental design. The rats were randomly divided into four groups of ten rats each:

1. Control group (n=10): no treatment.

2. MYC group (n=10): 5 mg/kg myricetin was injected to rats intraperitoneally per day for 7 days (Sun *et al*., 2018).

3. CP group (n=10): 7 mg/kg cisplatin was administered to the rats at 7th day of experiment (Ceylan *et al*., 2020).

4. MYC + CP group (n=10): 5 mg/kg myricetin was injected to rats intraperitoneally per day for 7 days and 7 mg/kg cisplatin was administered intraperitoneally to animals on 7th day.

During the injection period, the health status of the animals was checked twice a day and the changes in the health status of the animals due to myricetin injection were noted. Animals were anesthetized with a mixture of 30 mg/kg ketamine and 4 mg/kg xylazine after the injections (Fig. 1), and testis tissues were extracted from the animals for the histological and immunohistochemical analyses before the animals were euthanized.

Histological evaluation. The testis tissues were examined histologically by using conventional histological methods. Samples were fixed in 10 % formaldehyde for 24-48 hours, dehydrated using a series of alcohols, cleaned with xylene, and then embedded in paraffin blocks. They were then cut into 5 μm thick sections.

Hematoxylin-eosin (H&E) staining. Hematoxylin and eosin (H&E) staining was used to analyze the testicular tissue to evaluate the histopathological changes. By light microscopy (Leica DM IL LED, Leica Microsystems, Germany), pictures were taken and processed. The research team evaluated the testis tissue structure.

Histopathological score. Based on a scale of 1 to 10, the histological changes in the testicular tissues of the experimental groups were scored using Johnsen's Testicular Biopsy Score (JTBS). Two independent histologists evaluated, scored, and analyzed 100 seminiferous tubules for each group. In this scoring method, each seminiferous tubule is evaluated and givena score from 1 to 10, and the damage in the spermatogenic cell line is shown. The criteria employed by Johnsen to score the seminiferous tubules are listed in Table I (Akin *et al*., 2022).

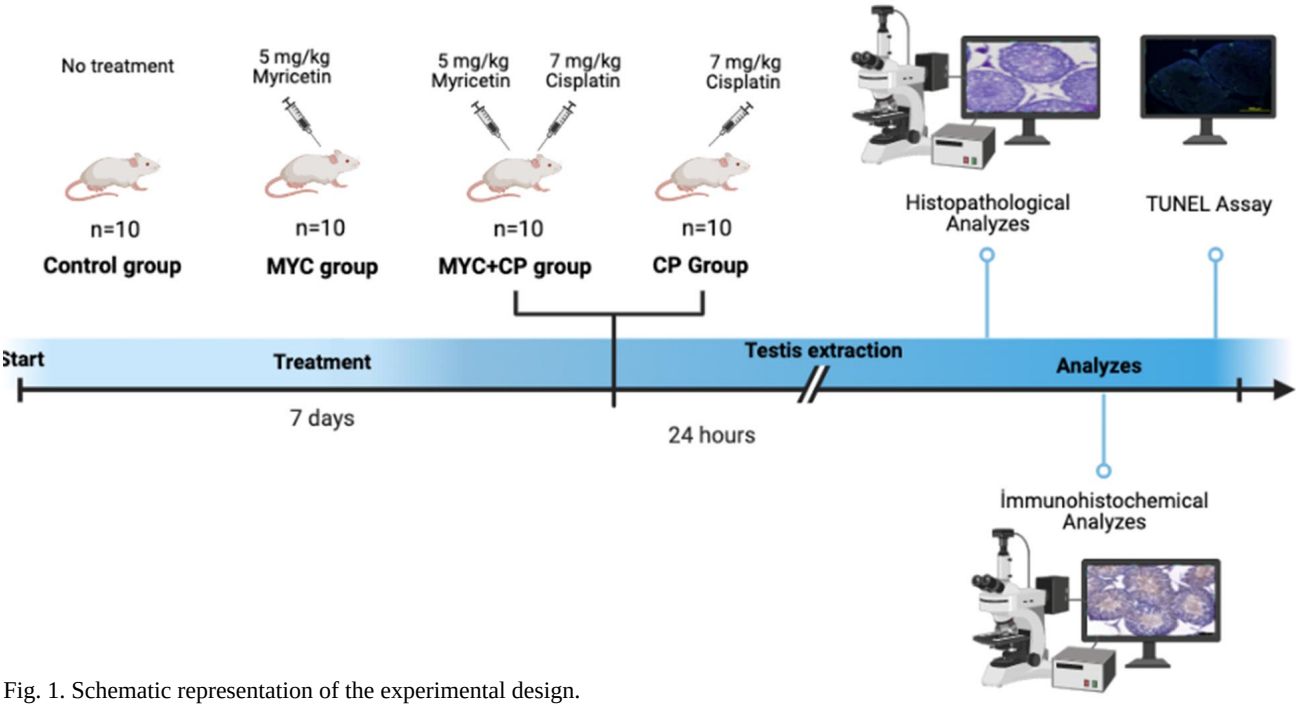

Fig. 1. Schematic representation of the experimental design.

Table I. Johnson's testicular biopsy scoring criteria for the evaluation of testicular damage.

Score	Histological findings
10	Complete spermatogenesis, numerous spermatozoa, germinal epithelium of regular height, tubular lumen of normal diameter
9	Numerous spermatozoa, germinal epithelium disorganized with sequestration of germinal cells, ubular lumen obturated
8	Less than 5 ± 10 spermatozoa per tubular cross-section
7	No spermatozoa, numerous spermatids, spermatocytes and spermatogonia
6	No spermatozoa, 5 ± 20 spermatids, numerous spermatocytes and spermatogonia per cross-section
5	No spermatozoa and spermatids, numerous spermatocytes and spermatogonia
4	No spermatozoa and spermatids, less than 5 spermatocytes, but numerous spermatogonia per cross-section
3	Only spermatogonia
2	No germinal cells, only Sertoli cells
1	No cells at all within the tubules

Measurement of the mean seminiferous tubule diameters (MSTD). Image J (1.45s, National Institute of Health, USA, RRID: SCR 003070) software was used to estimate mean seminiferous tubule diameters (MSTD) in micrometers. To determine the differences among the experimental groups in terms of MSTD measurement, at least 60 seminiferous tubules were chosen and measured.

Immunohistochemistry. Immunohistochemical approach was used to assess the changes in the expression levels of TLR4, NF-κB, HSP70, and HSP90 antibodies according to past research conducted by our research team (Sönmez *et al*., 2017; Kaymak *et al*., 2021). The paraffin blocks were cut into 5 μm sections. The tissues were deparaffinized with xylene and hydrated with an alcohol series. For antigen retrieval, sections were heated in a microwave at 350 W in a sterile urine container with 0.01 M citrate buffer. Sections were washed three times with phosphate-buffered saline (PBS) for 5 min each time. The slices were treated with %3 (w/v) H_2O_2 for 10 min to reduce endogenous peroxidase activity. The sections were treated with Ultra V Block solution after being rewashed three more times with PBS and kept in the incubation tank for 5 min. TLR4 (Anti TLR4 antibody, bs-20594R, Bioss, USA), NF-κB (Anti NF-κB antibody, FNab10334, FineTest, China), HSP70 (Anti HSP70 antibody, E-AB-64393, Elapscience, USA) and HSP90 (Anti HSP90 antibody, E-AB-60131, Elapscience, USA) antibodies diluted in proper ratios according to manufacturer datasheet were then applied to the tissues overnight at 4 °C. For the negative control experiments, PBS was applied the sections instead of primary antibodies after the sections were exposed to blocking serum, Ultra V Block. The remaining steps proceeded in the same way for both tissues exposed to the primary antibody and tissues subjected to negative control tests. The following morning, sections were rewashed three times with PBS before being incubated for 10 min with the secondary antibody (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA). The immunoreaction was amplified using streptavidin–avidin–peroxidase solution after rewashing with PBS, and the small intestine sections were seen with 3,3-p-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA). The images were captured using a light microscope. At least ten randomly selected fields on each section were scored at a 20x magnification. Image J (1.45s, National Institute of Health, USA, RRID: SCR 003070) software was used to measure the density of immunoreactivity (Karabulut *et al*., 2021).

Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling (TUNEL) assay. According to the instructions of the kit's manufacturer (Roche, In Situ Cell Death Detection Kit), the TUNEL assay was applied to evaluate apoptosis in testicular tissue. Antigen retrieval was achieved by placing sections in a sterile urine container with 0.01 M citrate buffer and heating them in a microwave oven at 350 W. 450 μL of the purple-capped label solution and the blue-capped enzyme solution were combined after washing with PBS. For negatives, we utilized 50 μL of the remaining label solution. In the dark, these solutions are applied to the tissues, which are subsequently incubated for an hour at 37 degrees. After washing with PBS, sections covered with 4',6-diamidino-2-phenylindole (DAPI) at dark and TUNEL-positive cells were detected. A total of 150 microscopic areas were evaluated for each group, and statistical analyses were performed to compare the experimental groups (Karabulut *et al*., 2020).

Statistical analysis. GraphPad Prism v9.0 for MacOS was used to conduct all statistical analyses (GraphPad Software, La Jolla, California, USA). The normal distribution of the data was assessed using the D'Agostino Pearson omnibus test. One-way analysis of variance (ANOVA) and Tukey's post-hoc test was used to compare quantitative variables when they showed a normal distribution. When the data exhibited an abnormal distribution, the Kruskal-Wallis test

and Tukey's post-hoc test were used to compare the experimental groups. Data with a normal distribution were expressed using the mean of normalized data and standard deviation of the mean, whereas data with an abnormal distribution were expressed using the median and minimum-maximum values. The cutoff point for statistical significance was 0.05 ($p<0.05$).

RESULTS

MYC protects testicular tissue against CP-induced testicular damage. When testicular tissue sections were stained with hematoxylin-eosin and photographed, the CP group's testicular tissue showed a greater number of histological alterations than the Control group and MYC group. It is observed that the spermatogenic cell line decreased, the seminiferous tubule widths shrank, and cells began to stream into the lumen in the CP group. In addition to these histological findings, two independent histologists determined that Johnsen's Testicular Biopsy Score for the CP group was statistically lower than for the Control group and the MYC group. Seminiferous tubules were detected to be more similar to tissue morphology in the MYC+CP group as opposed to the Control and MYC groups. Also, it was discovered that the MYC+CP group's seminiferous tubule diameters dramatically increased in comparison to the CP group (Figs. 2 and 3). Table II shows the JTBS and MSTD data for all experimental groups.

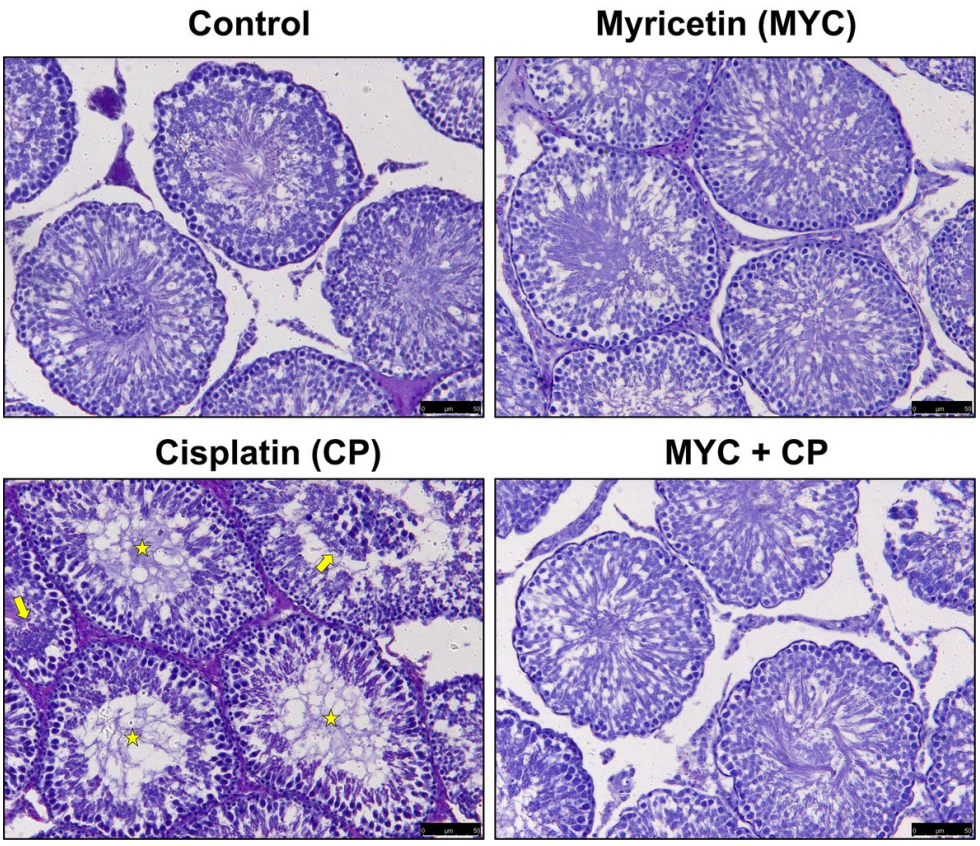

Fig. 2. Light microscopy of testicular tissue stained with H&E staining. Control group and MYC group showed normal histological tissue structure. In the CP group, damaged seminiferous tubules where spermatogenic cell line clearly reduced (stars) are seen. Moreover, this group showed the cells pouring into the lumen (yellow arrows). Unlikely, in the MYC+CP group, it is clearly seen the damage were substantially less when compared to CP group. Scale bar = 50 μm. Abbreviations: H&E, hematoxylin-eosin; CP, cisplatin; MYC, myricetin.

Table II. JTBS, MSTD and apoptotic cell number data of experimental groups.

Groups	Control	MYC	CP	MYC + CP	*p*
JTBS (1 to 10)	10 (8-10)	10 (8-10)	8 (6-10)*	10 (8-10)#	**0.001**
MSTD (μ)	264,4±29,0	264,9±25,7	250,5±20,4*	262,9±23,6#	**0.001**
Apoptotic cell number	0 (0-3)	0 (0-2)	0 (0-6)*	0 (0-2)#	

JTBS data are expressed as median (min – max), and MSTD data are expressed as mean±standard deviation. p <0.05 was considered as significant. * shows the statistically significant difference when compared with Control, MYC and MYC + CP groups. # shows statistically significant difference between CP and MYC + CP groups.

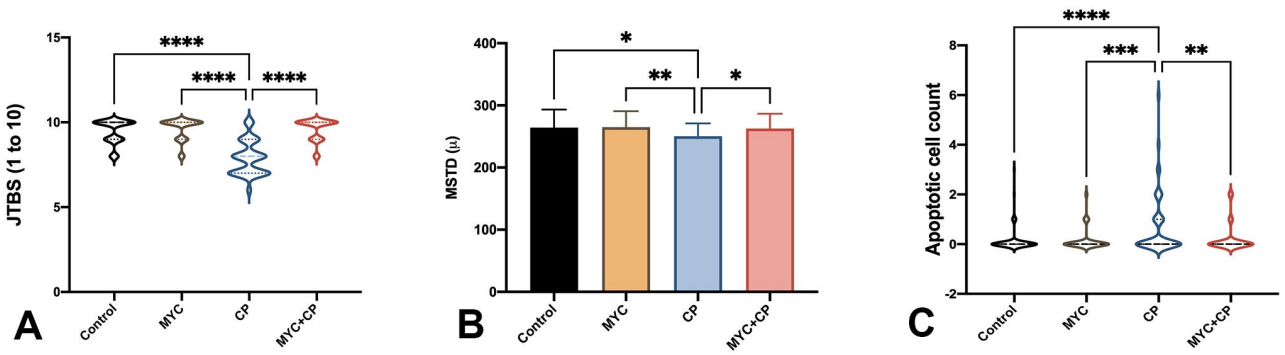

Fig. 3. Statistical analysis of JTBS (A), MSTD (B) and the number of apoptotic cells (C) in experimental groups. Control and MYC groups show higher JTBS and MSTD in testis sections; CP group shows significantly reduced JTBS and MSTD in the testicular tissue, suggesting serious damage in the testicular tissue. However, in the MYC+CP group, it is clearlyseen that the JTBS and MSTD data are significantly higher than those in the CP group and are like Control and MYC group. Moreover, apoptotic cell number in the CP group is significantly higher than those in the other experimental groups. Unlikely, it can be seen that MYC pretreatment significantly suppressed the induction of the apoptosis in the MYC+CP group. *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001. Abbreviations: CP, cisplatin; MYC, myricetin; JTBS, Johnsen's testicular biopsy score; MSTD, mean seminiferous tubule diameter.

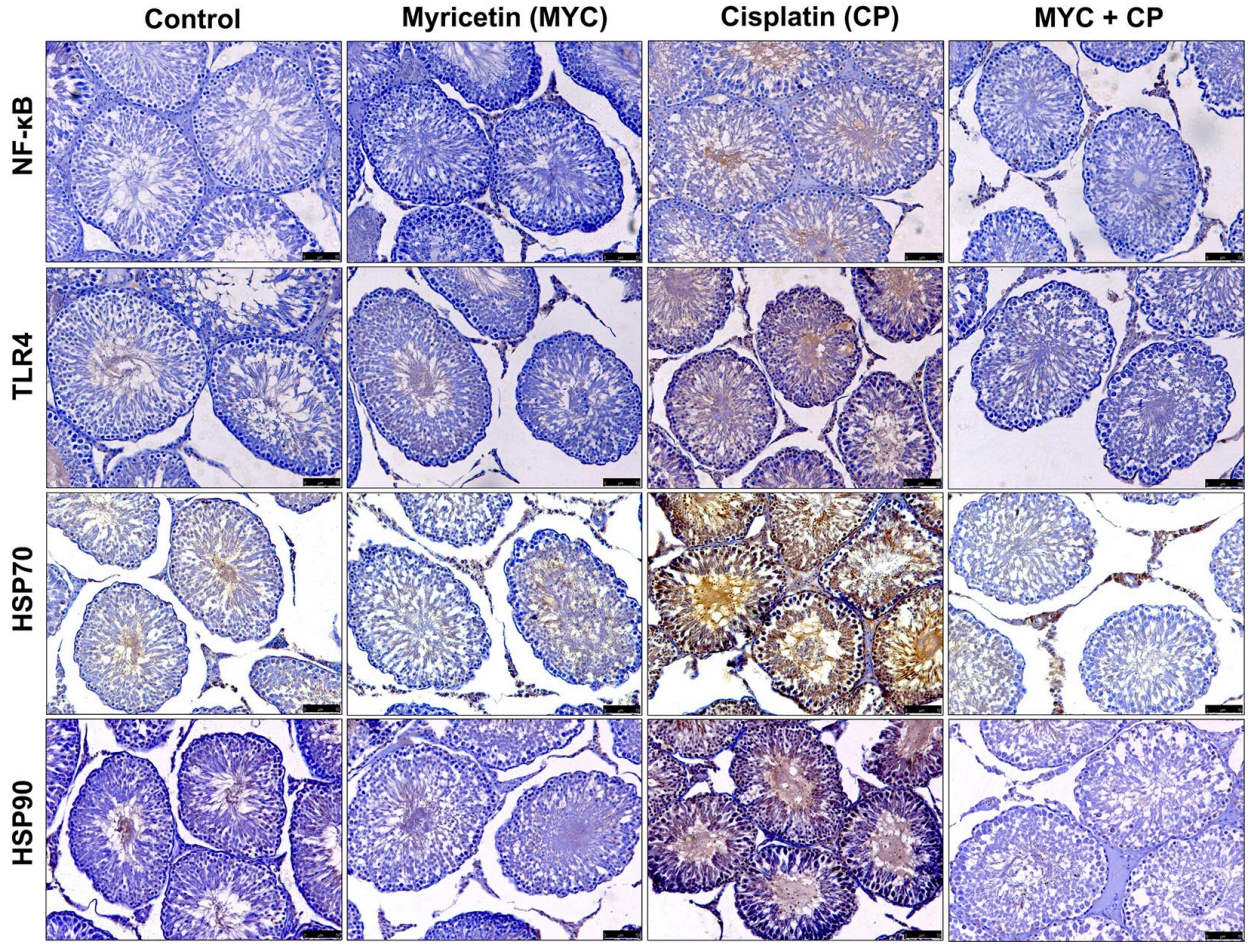

Fig. 4. TLR4, NF-κB, HSP70 and HSP90 immunostaining of testis tissues in experimental groups. Control and MYC groups show weak TLR4, NF-κB, HSP70 and HSP90 immunoreactivity in testis sections; CP group shows strong TLR4, NF-κB, HSP70 and HSP90 immunoreactivity. Unlikely, in the MYC+CP group, TLR4, NF-κB, HSP70 and HSP90 immunoreactivity were substantially less compared to those in the CP group. Scale bar = 50 μm. Abbreviations: CP, cisplatin; MYC, myricetin; NF-κB, Nuclear factor kappa B; TLR4, toll-like receptor 4; HSP70, heat-shock protein 70; HSP90, heat-shock protein 90.

TLR4/NF-κB pathway were not triggered thanks to MYC administrations. TLR4 and NF-κB immunoreactivity was present in experimental groups, according to the results of the immunohistochemical staining for TLR4 and NF-κB and statistical analysis of the immunoreactivity measurement data. However, in the Control and MYC groups, positively stained cells were hardly noticeable (Fig. 4). Our immunoreactivity density assessments using the Image J program revealed that the CP group had a considerably greater positive rate of TLR4 and NF-κB in the seminiferous tubules than the other experimental groups ($p<0.05$). Moreover, TLR4 and NF-kB expressions in the seminiferous tubules were considerably reduced in the MYC+CP group compared to the CP group ($p<0.05$), indicating that MYC inhibits the inflammatory response in the testicular tissue (Figs. 4 and 5A,B).

Inhibition of heat-shock response by MYC. According to the results of immunohistochemistry staining performed on testicular sections using HSP70 and HSP90 antibodies, the expression of HSP70 and HSP90 was immunohistochemically detected in the seminiferous tubules of the experimental groups (Fig. 4). The expression levels of HSP70 and HSP90 in the spermatogenic cell line were statistically higher in the CP group compared to the Control and MYC groups ($p<0.05$) based on immunohistochemical staining and measurements of the immunoreactivity. In contrast, the testicular sections of the MYC+CP group as compared to the CP group showed significantly lower expression levels of these variables ($p<0.05$). The immunoreactivity of HSP70 and HSP90 in testicular tissue is shown in Figures 4 and statistical analysis of the measures of immunoreactivity density in the spermatogenic cell line among the experimental groups is shown in Figures 5C and 5D.

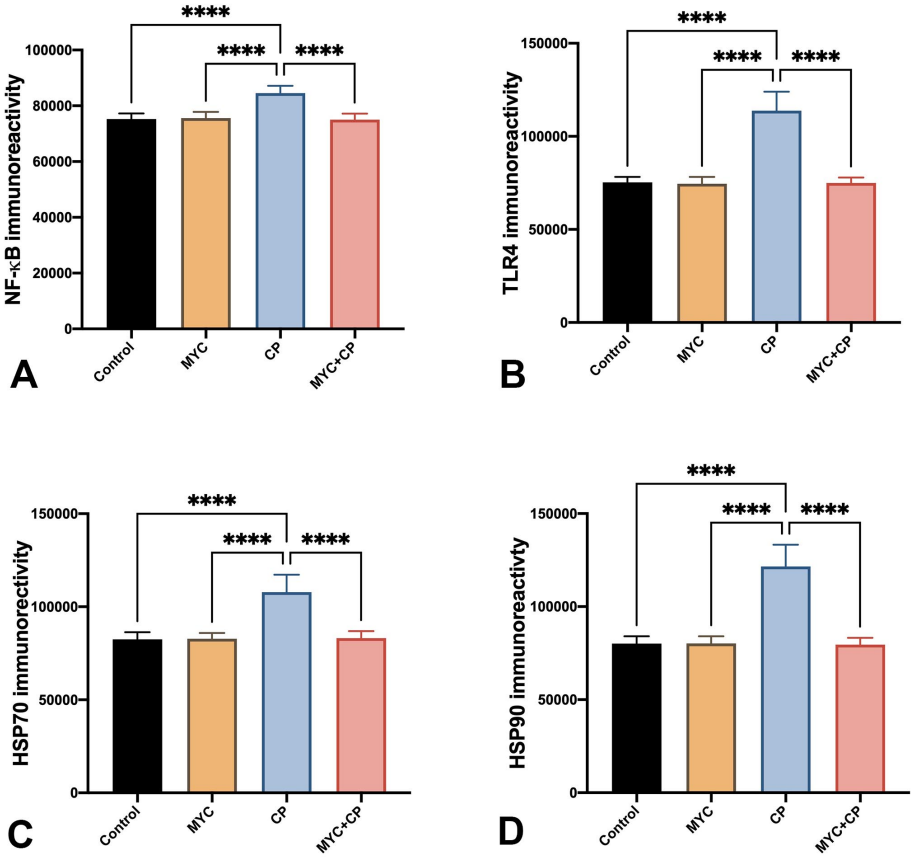

Fig. 5. Statistical analysis of the immunoreactivity measurements of the TLR4, NF-kB, HSP70 and HSP90 in the testis tissue sections of experimental groups. p <0.05 was considered as significant. The expression levels of TLR4, NF-κB, HSP70 and HSP90 were significantly higher in the CP group compared to Control and MYC groups ($p<0.05$). Moreover, MYC significantly inhibited the increased expression levels of the TLR4, NF-κB, HSP70 and HSP90 in the MYC+CP group when compared with the CP group ($p<0.05$). * shows the statistically significance among experimental groups. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$. Abbreviations: CP, cisplatin; MYC, myricetin; TLR4, toll-like receptor 4; NF-κB, Nuclear factor kappa B; HSP70, heat-shock protein 70; HSP90, heat-shock protein 90.

Anti-apoptotic activity of MYC against CP-induced apoptosis. By applying TUNEL assay, apoptotic cells in testicular tissue were detected (Fig. 6). The percentage of apoptotic cells in the testicular tissues was graded according to scoring system, based on 0-3 score. Minimum and maximum apoptotic cell number of Control and MYC groups was 0-3 and 0-2, respectively. Moreover, these two groups did not differ statistically. When compared to the Control and MYC group, the number of apoptotic cells increased in the CP group (0–6), which was statistically significant ($p<0.05$). The MYC+CP group had much less TUNEL-positive cells, and the number of apoptotic cells ranged from 0 to 2. The MYC+CP group showed considerably fewer apoptotic cells than the CP group ($p<0.05$). The statistical comparison of the number of apoptotic cells in the seminiferous tubules of the testicular tissues among experimental groups is shown in Figure 3C and Table II.

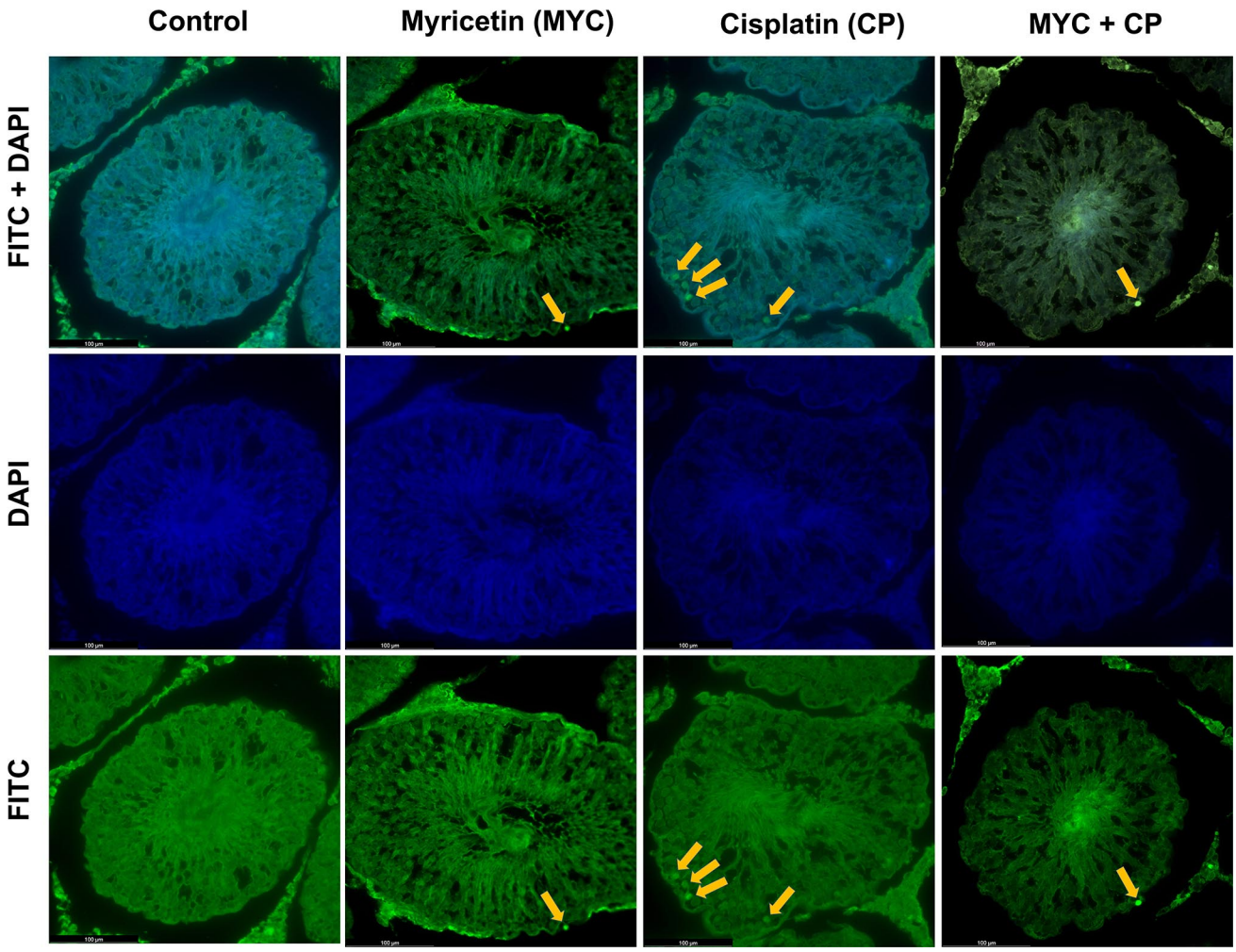

Fig. 6. TUNEL staining of testicular tissue sections. In the Control and MYC groups, normal testis tissues and a few apoptotic cells (yellow arrows) were observed. However, in the CP group TUNEL-positive cells (yellow arrows) were mainly observed in testicular tissue sections. On the contrary, in the MYC+CP group TUNEL-positive cells significantly decreased compared to CP group. Scale bar = 100 μm Abbreviations: TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, CP, cisplatin; MYC, myricetin.

DISCUSSION

Despite its potent anticancer properties, CP has a number of adverse effects, including ototoxicity, nephrotoxicity, neurotoxicity, testis toxicity, nausea, and vomiting, all of which can limit its use (Pasetto *et al*., 2006; Kim *et al*., 2018). Gonadotoxicity is a well-known dose-limiting adverse effect of CP that affects testis tissue and can result in azoospermia and low testosterone levels (Ciftci *et al*., 2011). Various processes, including oxidative stress, inflammation, and ischemic injury, have been implicated, albeit the mechanisms are not entirely understood (Singh *et al*., 2017). CP causes direct damage to spermatogenic cells and sustentacular cells (Sertoli cells) in the testes. It also causes interstitial cells (Leydig cells) to malfunction. Sperm production, seminiferous tubule diameter, and intratesticular testosterone level were found to be lower in CP-treated rats, but terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells increased (Yaman *et al*., 2018). Therefore, the most crucial aspect of CP chemotherapy is side effect protection.

Several studies have demonstrated that CP severely harms testicular tissue through a variety of events, including oxidative stress, inflammation, and apoptosis (A A Aly & Eid, 2020). In our study, the decrease in JTBS data showing an increase in histopathologic damage in the testicular tissue in the CP group and the narrowing of seminiferous tubule

diameters indicate that CP treatments cause serious damage to the testicular tissue. Since the mechanism of CP-induced injury in testicular tissue is known to be mostly related to inflammation, oxidative stress, and apoptosis, we propose that these histopathologic changes in testicular tissue are caused by CP-induced injury in testicular tissue in which multiple processes are involved. Furthermore, 5 mg/kg MYC pretreatment greatly reduced CP-induced damage in testicular tissue, indicating that this therapeutic agent exhibits significant protection in testicular tissue through its various biological properties. Therefore, we can say that the gonadoprotective effects of MYC is an effective protection in terms of histopathologic changes.

It is known that NF-κB, the main controller of inflammation, plays a critical role in testicular damage as a result of increased cytokine release and activation of protein kinases as a result of activation of via TLR4 and NF-κB (Kuzmich *et al*., 2017). Studies have shown that TLR4 and NF-kB expressions are significantly increased in CP-induced testicular injury (Ilbey *et al*., 2009; Hassanein *et al*., 2021). The fact that CP activates the TLR4/NF-κB pathway in testicular tissue was confirmed in our study by the increase in TLR4 and NF-κB expressions with CP administration. We think that the significant increase in TLR4 and NF-κB expressions in the CP group in our study is due to the fact that CP triggers severe inflammation in the testicular tissue. In addition, we found that 5 mg/kg MYC pretreatment suppressed the triggering of inflammation that caused severe damage to the testicular tissue. We suggest that this is because MYC has a potent anti-inflammatory effect at the dose of 5 mg/kg, protecting the testicular tissue from the destructive effects of CP-induced testicular damage.

HSPs are produced in a variety of cell types in the testicular tissue for rapid response to various stress situations like heat or chemical stress and various illnesses. Stress-induced HSPs are essential for preventing irreversible cellular protein damage by attaching to unfolded or misfolded proteins to postpone heat-induced denaturation and protein aggregation in many cells (Nixon *et al*., 2017; Santiago *et al*., 2020). HSP70 is a chaperone protein that has a range of intracellular roles, including directing the correct folding of newly generated natural proteins in renal tissue, mending misfolded proteins, and eliminating damaged proteins before they lose their function completely (Zhipeng *et al*., 2006). It also inhibits the phosphorylation of stress kinases including JNK and p38 MAPK, which helps to decrease inflammation and apoptosis (Chebotareva *et al*., 2017). HSP70 is connected to cell defense and survival and is elevated in CP-induced damage, according to many studies (Akin *et al*., 2021). In this study, we investigated HSP70-mediated stress response in CP-induced testicular injury. In line with our immunohistochemical results, we determined that increased HSP70 expression in CP-treated experimental animals indicates that CP induces a severe stress response in testicular tissue. In conclusion, we suggest that HSP70 expression in testicular tissue is increased in the CP-treated group to remove misfolded proteins caused by CP-induced oxidative damage and to ensure proper shaping of newly produced proteins. Furthermore, since HSP70 has been shown to prevent inflammation and apoptosis, we can conclude that cells in testicular tissue try to defend themselves by preventing inflammation and death caused by oxidative stress induced by CP. MYC, on the other hand, which was used as a protective agent in this investigation, had a significant protective impact on testis tissue at a dose of 5 mg/kg, causing HSP70 levels in the seminiferous epithelium to be comparable between the Control and MYC groups. We believe that this circumstance arose because of MYC's protective characteristic in testicular tissue, which inhibits protein misfolding and oxidative stress-induced inflammation and cell death.

When testicular tissue is injured, the expression of the chaperone HSP90 increases. It is well-recognized that this chaperone is essential for the elimination or repair of misfolded proteins (Samadian *et al*., 2021). In our study, we suggest that the significant increase in the HSP90 expressions in the CP group is due to protein misfolding induced by CP administration. That's why it was already expected that HSP90 expressions increase because of increased protein misfolding due to oxidative stress in the CP-administered group. However, we think that the protection provided by 5 mg/kg MYC pretreatment in the testicular tissue prevented the induction of HSP90 response, which is due to the strong protective effect of MYC in the testicular tissue. We propose that this protective effect of MYC protects the testicular tissue from protein misfolding because of CP-induced oxidative stress.

The number of apoptotic cells in the testicular tissues of experimental animals exposed to 7 mg/kg CP administrations in the CP group was much higher than in the other groups, according to our findings. These findings, which were obtained using the TUNEL staining method to quantify apoptotic cells in testicular tissue, reveal that CP administrations cause substantial damage to testicular tissue by inducing apoptosis. Furthermore, at doses of 5 mg/kg, MYC, a potent anti-inflammatory, antioxidant, and anti-apoptotic drug, dramatically inhibited the amount of apoptosis in the MYC+CP group. MYC appears to protect testicular tissue from the detrimental effects of CP by inhibiting of the induction of apoptosis, according to TUNEL staining results.

CONCLUSION

In conclusion, testicular tissues from the experimental animals were investigated in this study and it is observed that histopathological changes increased because of CP chemotherapy. The inflammatory response in testicular tissue is indicated by a significant increase in the expression of TLR4 and NF-κB. Also, we believe that the rise in misfolded protein due to oxidative stress caused by CP is the reason for the increased HSP70 and HSP90 expression. Reactive oxygen species, which produce oxidative stress by attacking DNA and causing apoptosis, are recognized to be linked processes. Consequently, this notion is supported by the result that received CP treatment had a much higher number of TUNEL-positive cells. It has been demonstrated in this work that 5 mg/kg MYC administrations have a significant protective effect on several parameters in testicular tissue. Our immunohistochemical and biochemical findings showed that MYC inhibited the inflammatory and heat-shock response in the testicular tissue, preventing these processes from leading to apoptosis and protecting the testicular tissue from the detrimental effects of CP treatments. According to our results, it should not be ignored in future clinical studies that MYC can be used as a protective agent at a dose of 5 mg/kg in the treatment of the side effects of CP chemotherapy.

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Informed consent. The experimental protocol performed in the present study was approved by the Erciyes University's Experimental Animal and Local Ethics' Committee with number 22/107/2022.

AKIN, A. T. Efectos gonadoprotectores *in vivo* de la miricetina sobre el daño testicular inducido por cisplatino mediante la supresión de la vía de inflamación TLR4/NF-κB y la respuesta al choque térmico. *Int. J. Morphol., 41(6)*:1870-1880, 2023.

RESUMEN: El objetivo de este estudio es revelar los efectos gonadoprotectores de la miricetina (MYC), que tiene muchas propiedades biológicas, sobre el daño testicular inducido por cisplatino (CP) en ratas. Para este propósito, se dividieron 40 ratas albinas Wistar macho en 4 grupos: Control (grupo que no recibió tratamiento), MYC (grupo que recibió 5 mg/kg/i.p de miricetina durante 7 días), CP (grupo que recibió 7 mg/kg/i.p de cisplatino al séptimo día) y MYC + CP (grupo que recibió 5 mg/kg/i.p de miricetina durante 7 días antes de la inyección de 7 mg/kg/i.p de cisplatino). Después de las administraciones, se extrajeron y procesaron tejidos testiculares de animales según el protocolo de procesamiento de tejidos. Se realizó tinción con hematoxilina y eosina para evaluar los cambios histopatológicos y se aplicó la puntuación de biopsia testicular de Johnsen (JTBS) y se midieron los diámetros medios de los túbulos seminíferos (MSTD) para comparar los grupos experimentales en términos de cambios histopatológicos. Además, los niveles de expresión de TLR4, NF-κB, HSP70 y HSP90 se detectaron mediante tinción inmunohistoquímica y se midió la densidad de inmunorreactividad para determinar la diferencia en los niveles de expresión de estos factores entre los grupos. Además, se detectó apoptosis testicular mediante el ensayo TUNEL. Los datos de JTBS y MSTD fueron significativamente más bajos en el grupo CP en comparación con otros grupos y las administraciones de MYC protegen significativamente el tejido testicular contra el daño inducido por CP. Además, las expresiones de TLR4, NF-κB, HSP70 y HSP90 y las células apoptóticas aumentaron significativamente en el grupo CP ($p<0,05$). Sin embargo, las administraciones de MYC ejercieron un fuerte efecto gonadoprotector sobre el tejido testicular en términos de estos parámetros en el grupo MYC+CP ($p<0,05$). Según nuestros resultados, sugerimos que MYC puede considerarse como un agente protector contra el daño testicular inducido por cisplatino.

PALABRAS CLAVE. Cisplatino; Respuesta al choque térmico; Inflamación; Miricetina; Daño testicular.

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