



ARAŞTIRMA / RESEARCH

Histological evaluation of the effects of rapamycin and 3-methyladenine on cisplatin-induced epididymal injury in rats

Sisplatin-indüklenmiş sıçan epididimis dokusunda rapamisin ve 3-metiladeninin etkilerinin histolojik olarak değerlendirilmesi

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Abstract

Purpose: In this study, we aimed to determine the effects of autophagy inhibitor and activator on Cisplatin (Cis)-induced tissue damage.

Materials and Methods: A total of 24 male Wistar albino rats were divided into 4 groups including 6 rats per group in this study. Groups are as follows; Control, Cisplatin (Cis) (8 mg/kg), Rapamycin (Rapa) (2 mg/kg), 3-methyladenine (3-MA) (15 mg/kg). Rapa and 3-MA were given intraperitoneally for 15 days. Cis was administered as a single dose on the 7th day of the experimental period. At the end of the experimental procedure, epididymis tissues were extracted. Hematoxylin and eosin staining and Heat shock protein-70 (HSP70) immunohistochemistry were applied to the sections taken after histological techniques.

Results: Dispersion in the tubule basement membrane and vacuolization in the tubule was observed in the Cis group. It was also observed that some epithelial cells were more eosinophilic in the Cis group. Tissue sections of Rapa and 3-MA had a more regular appearance in terms of epithelization and tubule basement membrane. HSP70 immunoreactivity was observed in the intertubular connective tissue of all groups.

Conclusion: The epididymis was affected by agents such as Cis in terms of the protection of semen quality and potency of spermatozoa. Rapa may be more effective than 3-MA in the epididymis against Cis toxicity.

Keywords: Autophagy, cisplatin, epididymis, rapamycin, 3-methyladenine.

Öz

Amaç: Bu çalışmada sisplatin (Cis) doku üzerinde neden olduğu hasar üzerinde otofaji aktivatörü ve inhibitörü etkilerinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmada her grupta 6 hayvan olacak şekilde 24 adet Wistar albino cinsi erkek sıçan kullanıldı. Gruplar; Kontrol grubu, Sisplatin grubu (Cis) (8 mg/kg), Rapamisin (Rapa) grubu (2 mg/kg), 3-metiladenin grubu (3-MA) (15 mg/kg). Rapa ve 3-MA intraperitoneal 15 gün boyunca, Cis tek doz deneyin 7. Günü uygulandı. Deney sonunda epididimisler alındı. Histolojik teknikler sonrası alınan kesitlere Hematoksilen-Eozin ve Heat-shock-protein70 (HSP70) immunohistokimyası uygulandı.

Bulgular: Cis kesitlerinde tübül bazal membranında dağılıma, tübül içinde vakuolizasyon gözlemlendi. Ayrıca bazı epitel hücrelerinin daha eozinofilik olduğu gözlemlendi. Rapa ve 3-MA ait doku kesitleri epitelizasyon, tübül bazal membranı olarak daha düzenli bir görünüme sahipti. HSP70 immunreaktivitesi tüm grupların tübüler arası bağ dokusunda gözlemlendi.

Sonuç: Semen kalitesi ve spermatozoanın potansiyelliğinin korunması için organın Cis gibi ajanlardan etkilendiği sonucuna varılmıştır. Rapa, Cis toksisitesine karşı epididimis üzerinde 3-MA'den daha etkili olabilir.

Anahtar kelimeler: Epididimis, otofaji, rapamisin, sisplatin, 3-metiladenin

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INTRODUCTION

The male genital system is a system consisting of the canal system and related organs that work in a regular and functional whole. Dysfunction in this canal system, where organs such as the testis and epididymis are located for healthy sperm production, can cause infertility. The epididymis is the organ where functional competence such as the ability to move, capacitate and interact with the zona pellucida is acquired, maturation and storage of spermatozoa passing from the testis to the excretory ducts¹. Changes in the epididymis may affect normal sperm physiology and thus inhibit sperm maturation². Nowadays, it is known that the male genital system is highly sensitive to various toxic agents, drugs, stress, and diseases. For this reason, it is necessary to determine how and to what extent the epididymis tissue is affected by these agents and diseases.

Cisplatin or cis-diamminedichloroplatinum(II) is a platinum-based antineoplastic drug that is widely used in the treatment of various malignancies, including testicular cancer. Despite its therapeutic importance, its usage is limited due to its side effects such as ototoxicity, hepatotoxicity, nephrotoxicity, and reproductive toxicity. After Cis treatment, the number of spermatozoa produced in the patients, their motility and vitality are reduced. There is also an increase in the number of morphologically abnormal spermatozoa^{3,4}. Besides the testis, based on an increase in dose, epididymal weight, and epididymal sperm number and motility is decreased after treatment⁵. It has been reported that the cytoprotective effects of molecular chaperones known as heat shock proteins (HSP) may be potentially important in the relationship of cells with Cis⁶. HSP70 is found in all subcellular compartments as a member of this molecular chaperone family⁷. It is known that HSP70 increases in the regulation of the disrupted physiological process in the cell after toxicity. Therefore, HSP70 will be an important marker in determining the damage due to Cis toxicity in the epididymis.

Autophagy is a process in which cells break down their own cytoplasm and organelles in lysosomes. The resulting breakdown products are used to generate energy and create new proteins and membranes. Autophagy maintains the health of cells and tissues by replacing old and damaged cellular components with new ones. Autophagy, a powerful

promoter of metabolic homeostasis, prevents degenerative diseases. However, it has a downside; cancer cells use it to survive nutrient-poor tumors⁸. Rapamycin (sirolimus) (Rapa), an antibiotic derived from *Streptomyces hygroscopicus*, is an FDA (U.S. Food and Drug Administration) approved immunosuppressant drug. Rapa targets various cellular functions such as cell growth, proliferation, and autophagic cell death and plays a critical role in the pathophysiology of cancer, diabetes, neurological disorders, and cardiovascular diseases⁹. Rapa is a specific mTOR inhibitor that can specifically bind to mTOR (mechanistic target of rapamycin) and disrupt its function. Thus, it activates the autophagy of cells by inhibiting mTOR and is also widely used as an autophagy activator^{9,10}. 3-Methyladenine (3-MA) is a phosphatidylinositol 3-kinase (PI3K) inhibitor that can affect the formation of autophagosomes and inhibit autophagy. Therefore 3-MA is also considered a mature autophagy inhibitor¹¹.

Various changes occur in the cell against cis toxicity, and this triggers various pathways in the cell. In this study, we focused on the relationship between Cis toxicity and autophagy. Therefore, we aimed to determine the extent to which the epididymis tissue is affected after possible Cis toxicity. In addition, we aimed to evaluate whether autophagy activator or inhibitor would be more effective against this toxicity in epididymis tissue, both histologically and HSP70 protein expression. Thus, the histological changes that occur in the epididymis tissue after Cis toxicity will be revealed and alternative treatment methods against the possible toxicity of Cis will be mentioned.

MATERIALS AND METHODS

Animals and drug administration

The study protocol was accepted by the Experimental Animal and Local Ethics Committee of Erciyes University (Decision no: 18/161, Date: 12.12.2018). *Wistar albino* rats were taken from the Erciyes University's Experimental Animal Laboratory. The ages of the animals were between 8 and 10 weeks. The animals were housed between 20 and 22°C under a 12 h light / 12 h dark cycle and were fed by ad libitum.

A total of 24 Wistar albino male rats were used in the study, with 6 animals in each group. Groups; Control group, Cis group (8 mg / kg)⁵, Cis+Rapa group (2 mg / kg)¹⁰, Cis+3-MA group (15 mg / kg)¹¹. While Rapa

and 3-MA were administered for 15 days, Cis was applied to these groups on the 7th day of a single experiment. At the end of the experiment, the organs of the rats, which were anesthetized, were removed and placed in formaldehyde for histological follow-up. Hematoxylin-Eosin staining and Heat-shock protein 70 (HSP70) immunohistochemistry were applied to the sections taken after histological techniques.

Hematoxylin and eosin staining

5 micrometer (μm) sections taken from paraffin blocks were spread on slides. Standard histological methods were applied to the slides prepared. Paraffin was removed with xylol and passed through graded alcohol series and diluted. Sections were stained with hematoxylin-eosin (HE) to see the general histological structure. Sections were examined after passing through increasing alcohol series and xylene. Hematoxylin and eosin were purchased from Nanotek Lab. The stained slides were then examined under an Olympus BX51 microscop¹².

Heat shock protein 70 (HSP70) immunoreactivity

Immunohistochemistry was applied to epididymis tissue sections to show HSP70 immunoreactivity. Sections were washed after being treated with xylene and alcohol. The antigen sites were exposed by applying citrate buffer. Sections treated with 3% H₂O₂ were washed with phosphate buffer saline (PBS). The next steps were performed according to the procedures of the immunohistochemistry staining kit Ultravision Detection System (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA). HSP70 (sc-33575, Santa Cruz, USA) was used as the primary antibody. For staining the antibody-dependent regions in the sections, 3,30-p-diaminobenzidine tetrahydrochloride (DAB) (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was used. Counterstaining was then done with Gill-hematoxylin. The sections passed through the alcohol and xylene series were closed and examined under a microscope¹³. Image J program was used for immunoreactivity measurements.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 21.0). Whether the data were

normally distributed was determined by both The Kolmogorov–Smirnov test and The Shapiro-Wilk normality test. In the case of normal distribution, quantitative variables were compared using one-way analysis of variance and Tukey's posthoc test. Kruskal Wallis test and Tukey's post-hoc test were used for comparing the quantitative with the abnormal distribution. The data were expressed as the mean of normalized data $\pm$ standard deviation of the mean. $p < 0.05$ was considered as statistically significant.

RESULTS

Light microscopic examination

Control tissue sections had a normal histological appearance (Figure 1A). Disorganization in the tubular cells and vacuolization in the tubule were observed in some areas of the cis sections, in the tubular basement membrane, and in the intertubular connective tissue. It was also observed that some epithelial cells stained more eosinophilic. In addition, there were vacuolization-like spaces in the tubular lumens where the spermatozoon should be (Figure 1B). Tissue sections of Cis + Rapa and Cis + 3-MA had a more regular appearance as epithelization, tubule basement membrane. Eosinophilic cells were observed in the tubule epithelization of Cis + 3MA (Figure 1D).

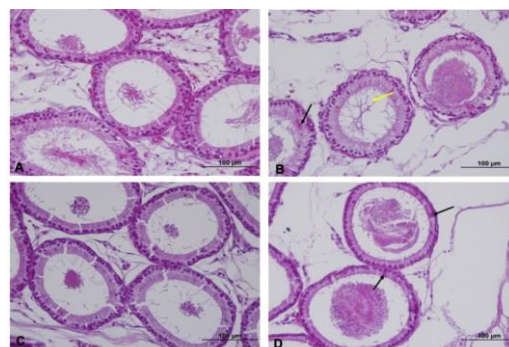


Figure 1. HE staining belonging to groups. A- Control group, B-Cis group, C-Cis+Rapa group, D-Cis+3-MA group. Eosinophilic cells (black arrows), vacuolization (yellow arrow). Scale bar 100 μm .

HE; Hematoxylin-Eosin, Cis; Cisplatin, Rapa; Rapamycin, 3-MA; 3-Methyladenine.

Heat shock protein 70 immunoreactivity

HSP70 immunoreactivity was observed in the intertubular connective tissue of all groups (Figure 2).

HSP70 immunoreactivity was observed in the tubular epithelium of both Cis + Rapa and Cis + 3-MA tissue sections, mostly in the Cis group (Figure 2). However, HSP70 immunoreactivity was higher in both epithelial tissue and connective tissue in Cis compared to Control (Figure 2A-2B) ($p < 0.0001$). Cis + Rapa HSP70 immunoreactivity was decreased in

both epithelial tissue and connective tissue compared to the Cis group (Figure 2B-2C) ($p < 0.0001$). Cis + 3-MA (similar to Cis group) HSP70 immunoreactivity was higher in both epithelial tissue and connective tissue compared to the control group (Figure 2B-2D) ($p < 0.0001$). Statistical evaluations are shown in Table 1 and Figure.3.

Table 1. HSP 70 immunoreactivity results

	Control	Cis	Cis + Rapa	Cis + 3-MA
HSP 70 epithelial immunoreactivity	103.54 $\pm$ 9.00 ^a	112.34 $\pm$ 5.22 ^b	106.02 $\pm$ 4.69 ^a	111.37 $\pm$ 8.95 ^b
HSP 70 connective tissue immunoreactivity	103.72 $\pm$ 11.85 ^a	112.47 $\pm$ 8.79 ^b	105.25 $\pm$ 6.92 ^a	115.06 $\pm$ 10.57 ^b

Data are expressed as mean $\pm$ standard deviation (SD). There is no significant difference between groups containing the same letter (a, b, and c). $p < 0.05$ was considered significant. $p < 0.05$ all groups compared to each other. Abbreviations: HSP70; Heat Shock Protein, Cis; Cisplatin, Rapa; Rapamycin, 3-MA; 3-Methyladenine. Values are given as mean $\pm$ SD. $p < 0.0001$

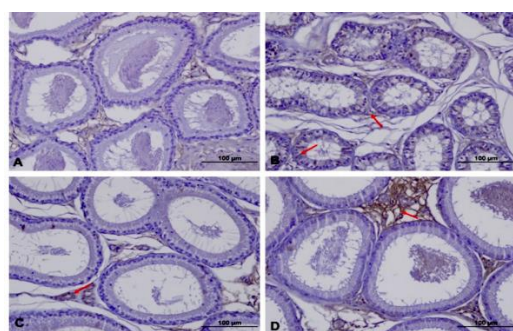


Figure 2. Epididymis tissue immunohistochemistry staining of all experimental groups. A-Control group, B-Cis group, C-Cis+Rapa group, D-Cis+3-MA group. HSP70 immunoreactivity (red arrows). Scale bar 100 μ m. HSP70; Heat Shock Protein, Cis; Cisplatin, Rapa; Rapamycin, 3-MA; 3-Methyladenine.

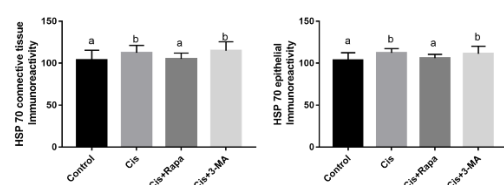


Figure 3. Graphs of HSP70 epithelial and connective tissue immunoreactivity results. There is no significant difference between groups containing the same letter (a, b, and c).

HSP70; Heat Shock Protein, Cis; Cisplatin, Rapa; Rapamycin, 3-MA; 3-Methyladenine.

DISCUSSION

Although Cis is an effective chemotherapeutic that can be used in many types of solid tumors, its obvious dose-dependent side effects such as testicular damage, azoospermia, oligospermia, and infertility limit its clinical use¹⁴⁻¹⁸. In addition to the therapeutic effects of Cis, its toxic effects extend to the auxiliary sex organs such as the epididymis and vas deferens and affect the sperm maturation events^{3,19}. It was observed that 8 mg/kg Cis dose administered in our study caused various histological changes in the epididymis, especially in the epithelium. Among them, disintegration in the tubular basement membrane and inter-tubular connective tissue, and large vacuolized areas in the lumen of the tubule. Spermatozoa passing from the testis to excretory channels; create a complex sequence of events that leads to the establishment of functional competence, such as the ability to act, capacitation, and interact with the zona pellucida. These functional changes occur in the epididymis during the post-testicular phase of sperm maturation. This sequence of events is the result of the secretory and absorbent activities of the epididymal epithelium. Cis-induced epithelial damage to the tissue may affect the physiological process. During their epididymal journey (transfer and storage), spermatozoa are at risk²⁰. For this purpose, preservation of the epididymal epithelium is important in maintaining normal physiology. It can be concluded that if this process is impaired, sperm transfer and storage function in the epididymis affected by Cis will be impaired.

Various processes play a role in the regular maintenance of cellular activity. These are important for the organ to fulfill its current task. One of these processes that take place inside the cell is autophagy. Autophagy is considered a process that plays an essential role in physiological and pathological conditions, preserved during evolution. Its main role is to eliminate harmful cytoplasmic components that are not needed, such as damaged organelles and poorly folded proteins. Thus, autophagy contributes to reducing the risk of formation of toxic protein clusters and supports cell survival. This catabolic process can be activated under various stress conditions or inhibited by various drugs²¹. Rapa, an immunosuppressor antibiotic, activates the autophagy of cells by inhibiting mTOR and is also widely used as an autophagy activator^{9,10}. Considered a mature autophagy inhibitor, 3-MA is a phosphatidylinositol 3-kinase (PI3K) inhibitor that can inhibit autophagy by affecting the formation of autophagosomes¹¹. With these properties, these substances, which are used as activators and inhibitors, can be used together to reduce the toxicity of drugs with various side effects and more effective treatment options can be revealed.

Cis is known to have different harmful effects in various systems. Bing Fang et al.²² reported that hearing loss and hair cell damage due to Cis-induced ototoxicity decreased with the application of 2 mg/kg Rapa. They reported that this decrease may be due to the decrease in MDA level and the decrease in oxidative stress and the increase of the expression of autophagy-related proteins LC3-II and Beclin-1. Bingying Wang et al.²³ showed that the same effect was imitated by Rapa (20 µg / kidney) in Cis-induced kidney damage similar to the protective effects of human umbilical cord MSC-derived exosomes (huc-MSC-ex). It has also been reported that the autophagy inhibitor 3-MA (500 µg / kidney) suppresses this protective effect. In different tissues, RP activates autophagy and reduces tissue damage; On the other hand, 3-MA has been reported to increase tissue damage by inhibiting autophagy. However, a comprehensive study has not yet been carried out on the effects of Rapa and 3-MA on Cis-induced epididymal tissue damage. For this purpose, by applying Rapa and 3-MA together with Cis in epididymis; Autophagy was activated and inhibited in the tissue, and how Cis affects the tissue was evaluated. When Rapa was applied with Cis, it had an appearance similar to the normal histological appearance in the epididymal tissue. However, in the

group where 3-MA was applied with Cis, the eosinophilic cells observed in the part facing the lumen were an indicator of tissue damage. Considering the histological changes in the cells, the presence of eosinophilic cells suggests that the necrotic process may be indicative.

HSPs, and particularly HSP70, act as molecular chaperones, helping proteins to fold and transport and their assembly into complexes, and protect cells from stress conditions²⁴. In our study, an increase in HSP70 immunoreactivity in the epididymis was observed after Cis application. We think that this increase is due to cell protection and recovery of impaired cellular activity due to Cis-induced stress in cells. At the same time, HSP 70 immunoreactivity increased in epididymis sections treated with autophagy inhibitor 3-MA. This increase may have increased in direct proportion to histological changes. Because we think that the inhibition of autophagy in the cell may have caused the cell's functionality to deteriorate, and when Cis toxicity is added, HSP70 immunoreactivity increases in order to restore tissue functionality. On the contrary, in the group in which autophagy was activated by Rapa, no statistically significant change was observed in HSP 70 immunoreactivity. Results are similar to the control group. Considering both histological and immunohistochemical findings, we can say that the activation of autophagy in the tissue contributed to the reduction of Cis-induced damage. Only histological determination of the relationship between Cis toxicity, Rapa and 3-MA is a limitation of the study. In the evaluation of epididymis function affected by Cis toxicity, methods such as biochemical or gene expression should be used and these findings should be supported.

As a result, it was concluded that the autophagy activator Rapa is more effective than the autophagy inhibitor 3-MA in the epididymal injury caused by Cis. In addition, we can say that molecular chaperones such as HSP70 contribute to this effect. Cis disrupts the normal ongoing physiological process of the cell and affects pathways such as autophagy. Rapa may be effective for the correction of impaired autophagy in the cell against Cis toxicity. Therefore, Rapa can be considered as a co-treatment option during Cis use. In addition to peripheral organ diseases, epididymis should be included among the harmful side effects of cis toxicity and its function should not be ignored.

Yazar Katkıları: Çalışma konsepti/Tasarımı: DK; Veri toplama: TC, DK, EÖ, ATA, EK, BY; Veri analizi ve yorumlama: TC, DK, BY; Yazı

taslağı: TC, DK; İçerğin eleştirel incelenmesi: TC, DK, BY; Son onay ve sorumluluk: TC, DK, EÖ, ATA, EK, BY; Teknik ve malzeme desteği: TC, DK, EÖ, ATA, EK, BY; Süpervizyon: DK; Fon sağlama (mevcut ise): yok.

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Ethical Approval: The experimental protocol performed in the present study was accepted by the Erciyes University's Experimental Animal and Local Ethics Committee with number Decision no: 18/161, Date: 12.12.2018.

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Conflict of Interest: Authors declared no conflict of interest.

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