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Effects of Curcumin on Changes in Spermatogenetic Cells of Rats Treated with Cisplatin

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Abstract: Cisplatin is an effective antineoplastic drug which is used to treat many types of cancer. The most common known side effect of this drug is infertility. Curcumin, also called turmeric, has antioxidant and antitumor activities. This study was designed to investigate the effects of curcumin on the changes in spermatogenetic cells caused by cisplatin, which is used as a chemotherapeutic drug especially in testicular cancers. For this purpose, randomly selected 36 Spraque Dawley rats were seperated to 4 groups that of control, cisplatin, curcumin and curcumin+cisplatin and each group was seperated to 3 subgroups, each of them have 3 rats, so total 12 subgroups were obtained. The testicular tissues obtained from rats were evaluated histochemically, immunohistochemically and ultrastructually. In the results of this study, it is observed that the rate of apoptosis increased significantly in the experimental groups given curcumin together with cisplatin injection, and the transition-1 protein which is involved in DNA packaging in the elongated spermatids belonging to stages of XII, XIII an XIV, immunopositivity is increased statistically in the cisplatin-administered experimental groups compared to control groups. Both light and electron microscopic findings showed that intense degeneration, vacuolization, germ cell loss and spermatogenetic arrest occurred especially in the group given curcumin together with cisplatin. The results of our study showed that cisplatin can cause problems in infertility through DNA packaging and dragging cells to apoptosis and necrosis. Physicians who use cisplatin for cancer treatment are advised not to use curcumin together with cisplatin because it exacerbates the side effects of the cancer drug.
Keywords: Apoptosis; cisplatin; curcumin; DNA packaging; spermatogenetic cell types; testis; transition protein-1

INTRODUCTION

Cisplatin (CIS) is an effective antineoplastic DNA-alkylating agent used to treat solid tumors of the testicles, bladder, ovary, cervix, endometrium, lung, head and neck (Colpi et al. 2004; Howell and Shalet 2005). Cisplatin interacts with DNA by forming in-chain and inter-chain cross-links. These cross-links cause a cytotoxic effect as they prevent the replication of DNA and the synthesis of RNA. Since the DNA to which cisplatin binds cannot be replicated again, the resulting DNA damage stimulates apoptosis. When this damage is too large to be repaired, it cannot be tolerated by the cell and causes cell death (Kanter et al. 2007).

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apoptosis in the tissue. In the study of Kuhlmann et al. In 1997(Kuhlmann et al. 1997), it was stated that cisplatin had primary and secondary effects. Cisplatin exerts its primary effect by causing inhibition of protein synthesis by causing nucleolar damage and ribosomal disorder, as well as glutathione (GSH) and protein-SH degradation in the cytoplasm and mitochondria. Its secondary effect is the inactivation of carrier proteins, lipid peroxidation; It shows through mitochondrial damage by causing Na +, K+, -ATPase inhibition, reduction in Ca++ uptake, ATP degradation, loss of membrane potential.

Experimental anticancer studies involving the toxicity of cisplatin on the reproductive system are limited (Cherry et al. 2004). The main side effects encountered during cancer treatment in humans are azoospermia and testicular atrophy. In animal models, it has been reported that cisplatin causes long-term damage in spermatogenesis, impairs testosterone secretion by inhibiting Leydig cells, and acute or chronic exposure to chemotherapy causes apoptosis in germ cells.(Ahmed et al. 2011) While Bax increased in testes, BCL2 decreased with cisplatin administration. Spermatogenesis is more sensitive to cisplatin than other chemotherapeutic drugs. Cisplatin induces lipid peroxidation by decreasing testicular antioxidant level (Ahmed et al. 2011; Turk et al. 2011). A decrease in the number of seminiferous tubules, atrophy in the tubules, degeneration, vacuolization in spermatogonia, decrease in the number of germ cells and thinning of the seminiferous tubule epithelium, a decrease in Johnsen's testis scoring are observed in the testes histopathologically (Turk et al. 2011).

Curcumin (CMN) is a yellow pigment found in castor saffron spice (Curcuma longa), also known as turmeric. Ilbey et al. (2009) showed that the removal of NF-kB (Nuclear Factor kappa B) activation by an antioxidant such as CMN is an effective method of protection in testicular damage caused by CIS. CMN has a strong protective effect on testicular damage caused by this agent and it is predicted that it may be beneficial in clinical practice. CMN has protective functions on testicular tissue in many pathological conditions (Giannessi et al. 2008; Kanter et al. 2007; Verma and Mathuria 2009; Wei et al. 2009). However, its molecular effects on normal tissues and cells have not been adequately studied.

The packaging of DNA by nucleosomes in somatic cells is not sufficient for spermatozoon with much smaller nucleus. Therefore, spermatozoon chromatin enters the repackaging process during spermiogenesis. During the packaging process, somatic histones undergo methylation, phosphorylation, and ubiquitination steps. After these steps, somatic histones are replaced by testis-specific histone variants, testis-specific histone variants are replaced by transition proteins (TP). At the last stage of the packaging process, TP leaves its place to protamine proteins and chromatin is packaged by protamine proteins and this process is completed (Laskey et al. 1993; Miller et al. 2010). Xia et al. (2012) treated mouse spermatids with a histone acetylase (HAT) inhibitor, CMN. The results of this study showed that the inhibitory effect of CMN on the germ cell line is dose dependent. Therefore, it has been observed that the apoptosis of primary haploid spermatids increases after CMN treatment. As expected, the level of acetylated histone was down regulated. In addition, it has been observed that transcription is stopped in spermatids and the dynamics of chromatin dependent factors are disrupted.

As a result of the literature reviews, while there are a limited number of studies examining the effects of curcumin and cisplatin on spermatogenetic serial cells (Xia et al. 2012; Mercantepe et al. 2018) no study has been found examining its effects on DNA packaging and cell apoptosis. In the present study, it is aimed to investigate the

changes caused by cisplatin on spermatogenetic serial cells by histochemically, immunohistochemically, TUNEL and ultrastructural methods, and also to evaluate the effects of orally administered curcumin on these cells.

MATERIALS AND METHODS

Animals

Our experiments were carried out in Boğaziçi University Life Sciences and Technologies Application and Research Center Experimental Animal Production and Care Unit (Vivarium) in line with the approval received at the meeting of Boğaziçi University Institutional Animal Experiments Local Ethics Committee on 22.02.2016/1. Male rats of 8-10 weeks of age with an average weight of 250-300 g belonging to Sprague Dawley strain were used in our study.

Experimental Design

Four groups were formed as control, cisplatin (CIS), curcumin (CMN) and cisplatin + curcumin (CIS + CMN). Each group was divided into three subgroups, resulting in a total of 12 subgroups. A total of 36 rats, 3 in each subgroup, were used in the study.

In the control group, 14 ml / kg saline was given intraperitoneally (IP) only on the first day of the experiment. The other subgroup was given 6 ml/day corn oil by gavage for 15 days. In the last subgroup, 14 ml / kg of saline was given by ip on the first day, and 6 ml / kg / day corn oil was given by gavage for 15 days. The rats in the control group were euthanized with CO_2 gas on the 16th day and testicular tissues were removed. Rats in the CIS group were given a single dose of 7 mg / kg cisplatin (14 ml / kg from a 100 ml ready to use solution containing 50 mg active substance; KOÇAK Barcode no: 8699828770077) on the first day of the experiment.(Atessahin et al. 2006; Türk et al. 2008) Subgroups in the CIS group were euthanized with CO_2 gas on days 6, 11, and 16, respectively, and testicular tissues were removed.

Rats in the CMN group were given 200 mg / kg of CMN (Curcumin Sigma C1386) dissolved in 6 ml / kg / day corn oil by gavage for 5, 10, 15 days according to the experimental group(Chuang et al. 2000; Bayrak et al. 2008), respectively. On the 6th, 11th and 16th days, testicular tissues were removed by euthanasia with CO_2 gas. The rats in the CIS + CMN group were administered a single dose of 7 mg / kg CIS intraperitonally on the first day of the experiment, and according to the experimental group, 200 mg / kg curcumin was dissolved in 6 ml / kg / day corn oil and administered by gavage for 5, 10, 15 days. The rats in this group were euthanized with CO_2 gas on days 6, 11 and 16, respectively, as in the other groups, and testicular tissues were removed. 3 animals in each group were weighed before and after the experiment. Right and left testes of 3 animals in each group were weighed and weight averages were taken.

Histological Preparation, Histopathological and Histometrical Analyses

Left testis tissue taken from rats was fixed in Bouin’s solution for 24 hours for light microscopy and was embedded in paraffin after routine tissue follow-up steps using an automated tissue processor. The 5µm thick sections taken were evaluated histologically after hematoxylin-eosin (H&E) staining. Spermatogenesis was evaluated histopathologically using the Johnsen mean testicular biopsy score (MTBS) criteria.(Johnsen 1970; Aktas et al. 2012) Each animal was given a score of 0-10 according to these criteria in 3 slides. In the groups, 10 round or nearly-round seminiferous tubulus diameters, randomly selected from each animal, were measured (Songur et al. 2016). Measurements were carried out interactively with the help of the

Olympus BX43F research microscope and the CellSens Entry Image Analysis Program.

Immunohistochemistry

Transition protein-1 (TP 1) which is the intermediate protein between histone-protamine proteins during DNA packaging process in spermatogenesis is labeled by rabbit TNP1 / TP1 polyclonal primary antibody (LifeSpan Biosciences LS-B11624) antibody immunohistochemically. For this purpose, the right testis of each rat was fixed in 10% neutral buffered formaldehyde solution for immunohistochemical examination and was embedded in paraffin after routine tissue follow-up steps using an automated tissue processor. Two sections of 5 µm thickness were taken from each animal's block onto Poly-l-lysin coated slides. The sections were boiled in boiling antigen retriaval (0.1 M tri-sodium citrate dihydrate) for 5 minutes after deparaffinization and dehydration processes. The sections washed with phosphate buffer (PBS) were kept in 3% H_2O_2. After washing with PBS, it was incubated with blocking serum (Invitrogen, Blocking Solution Lot No: 1666262A) at room temperature.

Except negative slides, sections were treated with 1/250 diluted primary antibody and all slides were incubated overnight at + 4^oC in humidity chamber (LifeSpan Biosciences, LS-B11624). After washing with PBS, the secondary antibody (Invitrogen, Broad Spectrum Second Antibody Lot No: 1666262A) was dropped onto the slides and incubated for 1 hour in humidity chamber at 37^oC. After washing with PBS, HRP (Invitrogen, HRP-Streptavidin Lot No: 1666262A) was dropped onto each section and incubated for 1 hour in a 37^oC oven. After washing the slides with PBS, DAB was dropped for coloring. After nucleus staining with hematoxylin, the sections were covered with entellan and prepared for evaluation. Elongated spermatids belonging to stage XII, XIII and XIV, in which TNP-1 is expressed, were evaluated and counted as immunopositive. For this, spermatids in 9 tubules were counted for each stage in each group, 3 in each slide of each animal. The percentage values of the counted spermatids were taken and compared statistically.

TUNEL

TUNEL kit (Merck Mlliopore, catalog number: S7100) was used in our study for the determination of apoptosis in cells. Two sections of 5 µm thickness were taken from the tissues fixed in 10% neutral buffered formaldehyde of each animal. After deparaffinization and dehydration, the sections treated with Proteinase K were kept in H_2O_2 at room temperature. The slides washed in PBS were kept in the equilibration solution and then TdT enzyme was applied. Preparations that were shaken quickly in the stop / wash solution were incubated in the anti-dioxygenin peroxidase (APK) conjugate in humidity chamber. After washing in PBS, DAB was dropped for coloring and staining was done. After nucleus staining with hematoxylin, slides were covered with entellan and prepared for evaluation.

The slides were examined under a light microscope (Olympus CX21) x40 objective. The reaction was considered positive if brown precipitation was seen. The number of apoptotic cells was calculated by counting the apoptotic cells seen in 100 tubules for each animal. In addition, the number of tubules containing at least one apoptotic cell in 100 tubules counted for each animal was determined and the TUNEL positive rate was calculated.

Electron Microscopy

Electron microscopic tissue processing and examination of our study was carried out in the Department of Histology and Embryology at Istanbul University, Istanbul Medical Faculty. Half of the left testicular tissues taken from one rat from the control and experimental groups (15 days grups) were kept in cacodylate buffered

2.5% glutaraldehyde solution for 1 day for electron microscopy study. Electron microscopic tissue process was performed on a Leica EM TP model tissue processor. Sections of 70 nm were taken on copper or nickel surface carriers called grid by Leica EM UC7 model ultramicrotome. After contrasting with uranyl acetate, sections were examined and photographed under electron microscope.

Statistical Analysis

Statistical analysis of the obtained data was carried out using the spesific data analysis computer program. The normal distribution relevancy of the data was done using the Shapiro Wilk Test. Normally distributed body weight and testicular weight data, % TUNEL positive tubulus ratio and seminiferous tubulus diameters were evaluated by One-Way ANOVA Test and post-hoc Duncan Test. Normal distribution fit of the data was done by Shapiro Wilk Test. Since histochemical and immunohistochemical data and the number of apoptotic cells per tubulus did not show normal distribution, the Kruskall Wallis Test was used, and the significance control of the difference between groups in the data where the intervention was important was performed using the Bonferroni-Corrected Man-Whitney-U Test.

Ethical Approval

Our experiments were carried out in Boğaziçi University Life Sciences and Technologies Application and Research Center Experimental Animal Production and Care Unit (Vivarium) in line with the approval received at the meeting of Boğaziçi University Institutional Animal Experiments Local Ethics Committee on 22.02.2016/1.

RESULTS AND DISCUSSION

Analysis of body weight, testis weight and Seminiferous Tubulus diameter

When the body weight averages of the groups were examined after the experiment, a statistically significant decrease in body weights was observed in the cisplatin and cisplatin + curcumin groups compared to the controls (Table 1). Testicular weight averages are summarized in table 1. On the 5th day of the application, it was observed that the testicular weights of the CIS-5, CMN-5, and CIS CMN-5 groups increased compared to the controls. Testicular weights of CIS KMN-10 and CIS CMN-15 groups were observed to decrease compared to controls. Tubulus diameters were found to be narrower in the CIS-10, CIS CMN-10 and CIS CMN-15 groups compared to the other groups, and this narrowing was statistically significant (Table 1).

Histochemical Findings

The comparison of the groups scored according to Johnsen criteria is summarized in table 2 ($p < 0.05$). According to the results of this table, CIS CMN-5, CIS CMN-10 and CIS CMN-15 groups were statistically different from all control groups ($p < 0.05$). The sections belonging to the control and experimental groups were staged and the structures of the spermatogenetic serial cells were examined. In the testicular sections of the control group, spermatogonia and Sertoli cells were located in the basal compartment of the seminiferous tubules, primary and secondary spermatocytes and round (early), elongating (middle), elongated (late) spermatids and spermatozoa were located in the adluminal compartment (Figure 1A). When the testis sections of the experimental groups that were found to be statistically significant according to Johnsen criteria were examined, spermatogenetic arrest was observed especially in the CIS CMN-10 and CIS CMN-15 experiment group. In the microscopic examinations, separation between cells due to cell loss, cell debris in the lumen were observed in the CIS CMN-10 experiment group (Figure 1B). When the seminiferous tubules belonging to the same experimental group were examined in detail, it was

noted that there was no continuity in the cells after round spermatids due to the spermatogenetic arrest (Figure 1B).

When the testicular sections of the CIS KMN-15 experimental group were examined, more dramatic findings were observed in parallel with the Johnsen criteria. In microscopic examinations belonging to this group, germ cell loss was quite remarkable compared to other groups. It was observed that spermatogenesis could progress up to spermatocytes (Figure 1C). On the other hand, apoptotic cells (Figure 1C, 1D) and multinucleated polymorphic giant cells (Figure 1D) were noted along with morphological disorders in the cells. In spermatocytes, dense appearance in chromatin (Figure 1D), shrinkage in the nuclei, irregular shape of the tunica albuginea layer setting tubular boundaries, and testicular atrophy due to tubular shrinkage (Figure 2) were observed.

Table 1. Body weight after experiment, right and left testes mean weight and Seminiferous Tubulus diameter

Group	Body weight after experiment	Right and left testes mean weight	Seminiferous tubulus diameter
		$(\overline{x} \pm S_{\overline{x}})$	
CIS-5	234,66±19,47^{c,d,e}	2,56±0,15^{d}	306,73±6,69^{d}
CMN-5	252,66±5,81^{d,e,f}	2,97±0,04^{e}	313,11±7,85^{e,d}
CIS KMN-5	210,66±2,9^{b,c,d}	2,43±0,13^{d}	299,21±4,88^{d}
CIS-10	226,93±7,13^{b,c,d,e}	1,82±0^{c}	277,12±4,08^{c}
CMN-10	230,69±20,75^{b,c,d,e}	1,55±0,1^{b,c}	304,20±5,49^{d}
CIS CMN-10	158,21±4,10^{a}	0,92±0,23^{a}	251,88±12,75^{b}
CIS-15	203,33±3,33^{b,c}	1,38±0,07^{b}	330,93±5,81^{e,f}
CMN-15	270,66±22,34^{e,f}	1,59±0,04^{b,c}	304,68±4,6^{d}
CIS CMN-15	187±20,59^{a,b}	0,89±0,25^{a}	210,93±6,83^{a}
Control Cg	282±7,21^{f}	1,58±0,02^{b,c}	340,53±6,6^{f}
Control Sp	294±6,11^{f}	1,72±0,09^{b,c}	310,87±10,93^{e,d}
Control Sp Cg	290±20,98^{f}	1,59±0,08^{b,c}	329,63±8,25^{e,f}

a,b,c,d,e,f : Groups with different letters in the same column are statistically significant ($p<0,05$). CIS 5: 5-day group given cisplatin, CMN 5: 5-day group given curcumin, CIS CMN-5: 5-day group given cisplatin and curcumin, CIS 10: 10-day group given cisplatin, CMN 10: 10-day group given curcumin, CIS CMN-10: 10-day group given cisplatin and curcumin, CIS 15: 15-day group given cisplatin, CMN 15: 15-day group given curcumin, CIS CMN-15: 15-day group given cisplatin and curcumin, Control Cg: Control group given corn oil, Control Sp: Control group given physiological saline, Control Sp Cg: Control group given saline and corn oil.

Table 1. Comparison Of Johnsen Criteria Scores Between Groups.

Group	Johnsen Score
	$(\overline{x} \pm S_{\overline{x}})$
CIS-5	8,55±0,17^{d,e}

CMN-5	9,11±0,2^{f,,g}
CIS CMN-5	8±0^{c}
CIS-10	8,22±0,14^{c,d}
CMN-10	8,88±0,11^{e,f}
CIS KMN-10	7,33±0,16^{b}
CIS-15	7,88±0,11^{c}
CMN-15	8±0^{c}
CIS KMN-15	5,11±0,48^{a}
Control Cg	9,66±0,16^{h}
Control Sp	9,44±0,17^{g,h}
Control Sp Cg	9,77±0,14^{h}

a,b,c,d,e,f,g,h: Groups with different letters are statistically significant (p<0,05). CIS 5: 5-day group given cisplatin, CMN 5: 5-day group given curcumin, CIS CMN-5: 5-day group given cisplatin and curcumin, CIS 10: 10-day group given cisplatin, CMN 10: 10-day group given curcumin, CIS CMN-10: 10-day group given cisplatin and curcumin, CIS 15: 15-day group given cisplatin, CMN 15: 15-day group given curcumin, CIS CMN-15: 15-day group given cisplatin and curcumin, Control Cg: Control group given corn oil, Control Sp: Control group given physiological saline, Control Sp Cg: Control group given saline and corn oil.

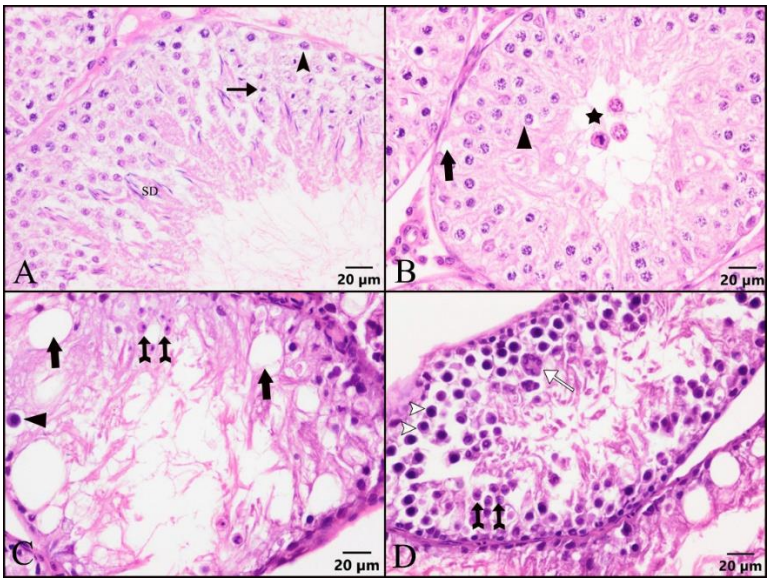

Figure 1. Seminiferous tubule sections belonging to Control sp cg (A), CIS CMN-10 (B), CIS CMN-15 (C, D) groups. In the control group, spermatogonia (arrowhead) in the basal compartment, meiotic spermatocytes (thin arrow) and elongated spermatids (SD) are observed in the adluminal compartment. In the experimental groups, cell debris (asterisk), intercellular separations (thick arrow), arrest in primary spermatocyte (triangle), apoptotic cells (double sided arrow), multinucleated polymorphic giant cell (white thin arrow) and dense appearance in chromatin (white arrow head) takes attention. Hematoxylin Eosin staining method, Bar: 20 µm.

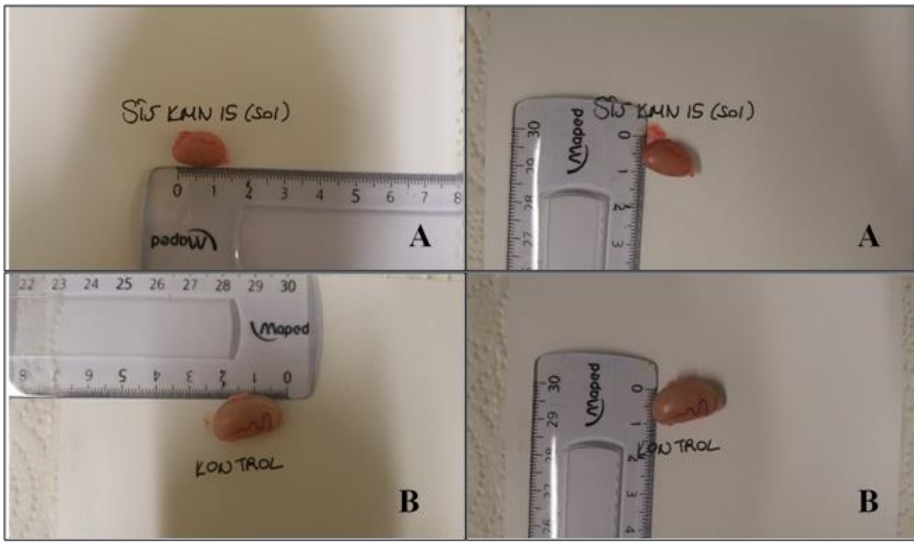

Figure 2. Atrophic testis. Left testis of an animal from the CIS CMN-15 group (A) appears to be atrophic compared to the control group (B).

Immunohistochemical Findings

TNP-1 expression was detected in the nucleus of the elongated spermatids belonging to stages XII, XIII and XIV. TNP-1 positive elongated spermatid rates belonging to stages XII, XIII and XIV is given in table 3. Since degeneration in the tubules was observed in the experiment groups of CIS CMN-10 and CIS CMN-15, it could not be separated into tubular stages and this was not taken into consideration. Control groups were combined because it was not statistically significant among themselves. When the groups are examined, stage XII immunpositive elongated spermatid rates were significantly increased in the CIS-5 experimental group (Figure 3) compared to the control (Figure 4) and other groups ($p < 0.05$) (Table 3). In general, it was observed that the rates of stage XII immunopositive elongated spermatid in other experimental groups were not statistically different compared to controls. However, in the groups given cisplatin, it was noted that after a significant increase in positive spermatids on the 5th day, it decreased severely on the 10th day and showed an increase close to the control values on the 15th day.

Stage XIII immunopositive elongated spermatid rates were found to be significantly increased in the CIS-5 experimental group (Figure 5) compared to the control groups (Figure 7) (Table 3). It was also noted that in the groups given cisplatin, after the significant increase in positive spermatids on the 5th day, it decreased on the 10th and 15th days and approached the value of the controls. However, interestingly, it was observed that in the group given only curcumin, the immunpositive cells increased on the 5th (Figure 6) and 10th days and decreased to values close to the controls (Figure 7) on the 15th day (Table 3). Stage XIV immunpositive elongated spermatid rates were observed to increase in the CIS-5 experiment group (Figure 8) compared to the control groups (Figure 9). It was also noted that in the groups given cisplatin, positive spermatid rates increased significantly on the 5th day and decreased on the 10th day, and on the 15th day it approached the value of the controls. Despite this fluctuation on the 10th and 15th days, it was not found statistically different (Table 3).

Table 3. Comparison of the Rates of Stage XII, XIII and XIV Positive Elongated Spermatids between Groups.

Group	Stage XII Positive	Stage XIII Positive	Stage XIV Positive
		$(\overline{x} \pm S_{\overline{x}})$	
CIS-5	80,71±4,18[a]	84,22±2,84[a]	80,92±2,4[a]
CMN-5	40,94±6,11[a,b,d]	58,26±7,16[a,b,e,g]	41,55±10,92[a,d]
CIS CMN-5	29,02±3,61[b,d]	36,75±6,31[a,c,e,f]	21,12±2,86[a,b,c,d]
CIS-10	13,45±3,16[b]	13,06±3,77[c]	1,4±1,4[b]
CMN-10	48,16±5,89[a,d,e]	56,28±5,38[a,d,g]	38,15±5,25[a]
CIS-15	46,64±5,89[a,d,f]	27,39±1,84[c,g]	19,29±1,25[a,b]
CMN-15	54,05±8,3[a,d,g]	20,88±2,50[c,d,e]	4,72±2,15 [b,c]
Control Groups	37,37±3,47[b,c,d,e,f,g]	23,67±3,16 [c,f]	10,47±2,01[b,d]

a,b,c,d,e,f,g: Groups with different letters in the same column are statistically significant (p<0,05). CIS 5: 5-day group given cisplatin, CMN 5: 5-day group given curcumin, CIS CMN-5: 5-day group given cisplatin and curcumin, CIS 10: 10-day group given cisplatin, CMN 10: 10-day group given curcumin, CIS 15: 15-day group given cisplatin, CMN 15: 15-day group given curcumin, Control groups: All control groups.

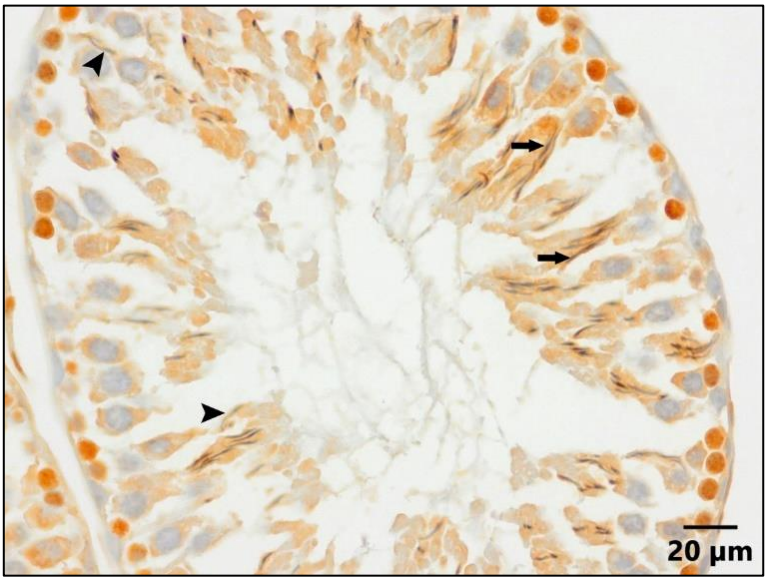

Figure 3. Tnp-1 expression in stage XII seminiferous tubule section of an animal from the CIS-5 experimental group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

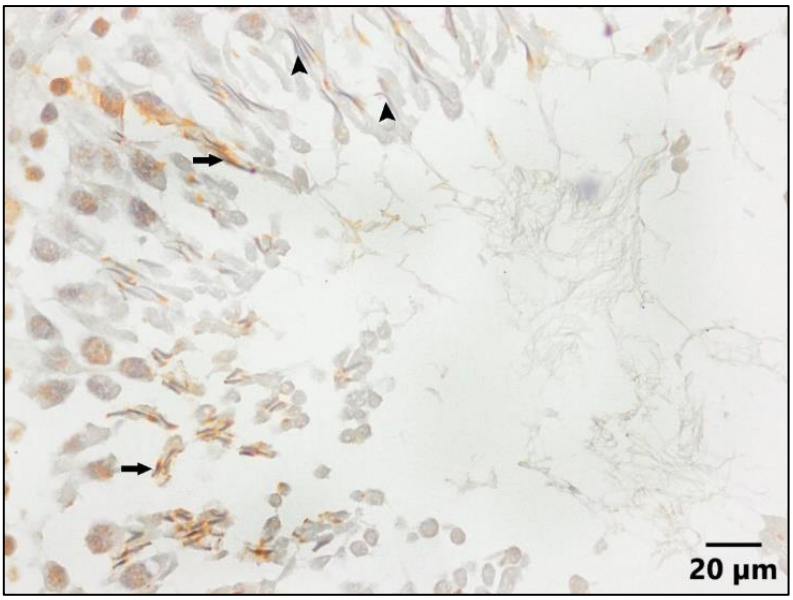

Figure 4. Tnp-1 expression in stage XII seminiferous tubule section of an animal from the control group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

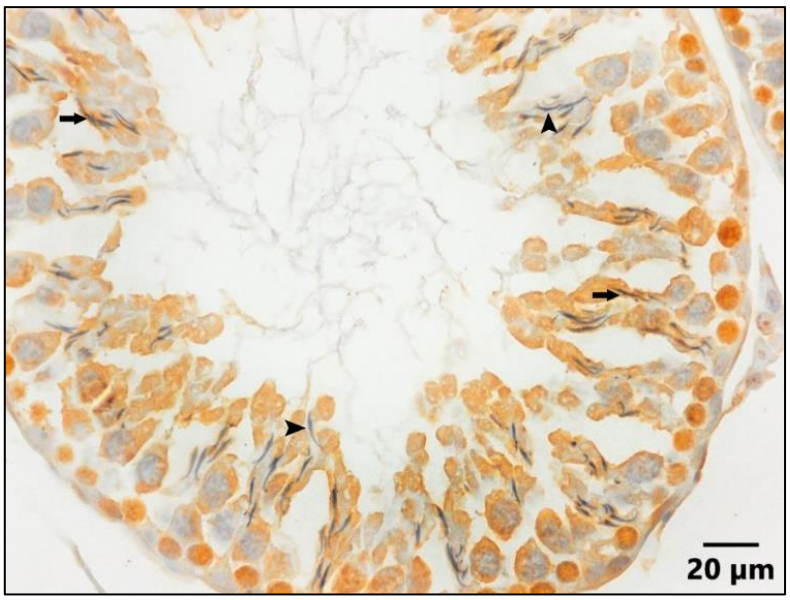

Figure 5: Tnp-1 expression in stage XIII seminiferous tubule section of an animal from the CIS-5 experimental group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

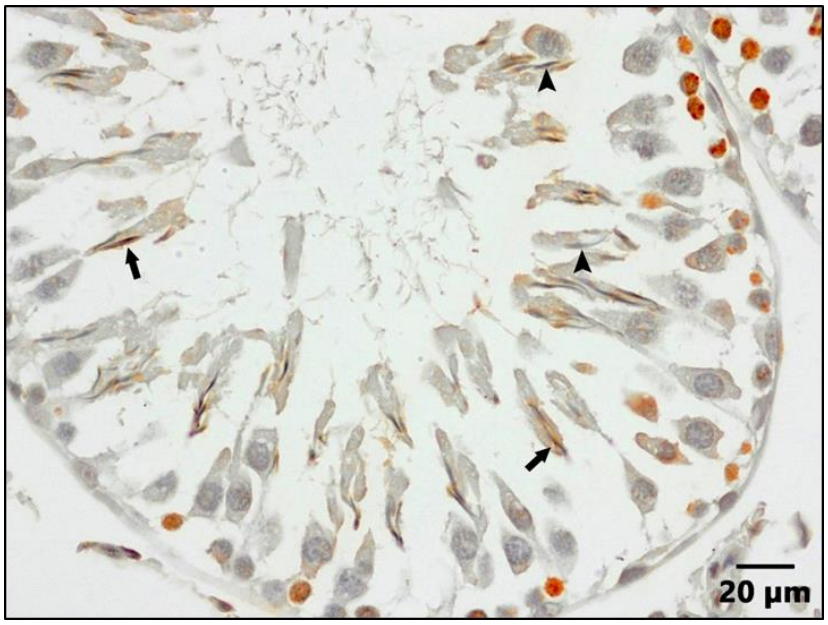

Figure 6. Tnp-1 expression in stage XIII seminiferous tubule section of an animal from the KMN-5 experimental group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

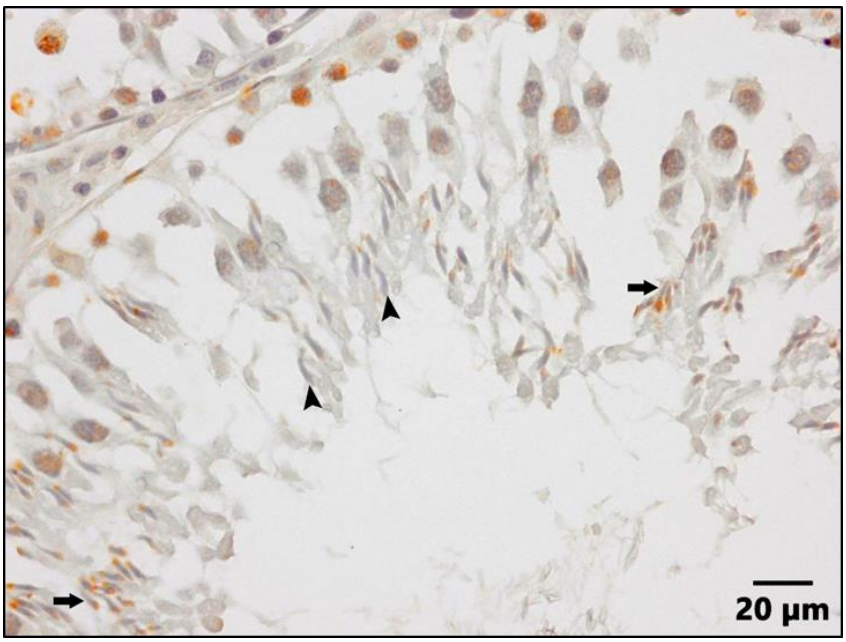

Figure 7. Tnp-1 expression in stage XIII seminiferous tubule section of an animal from the control group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

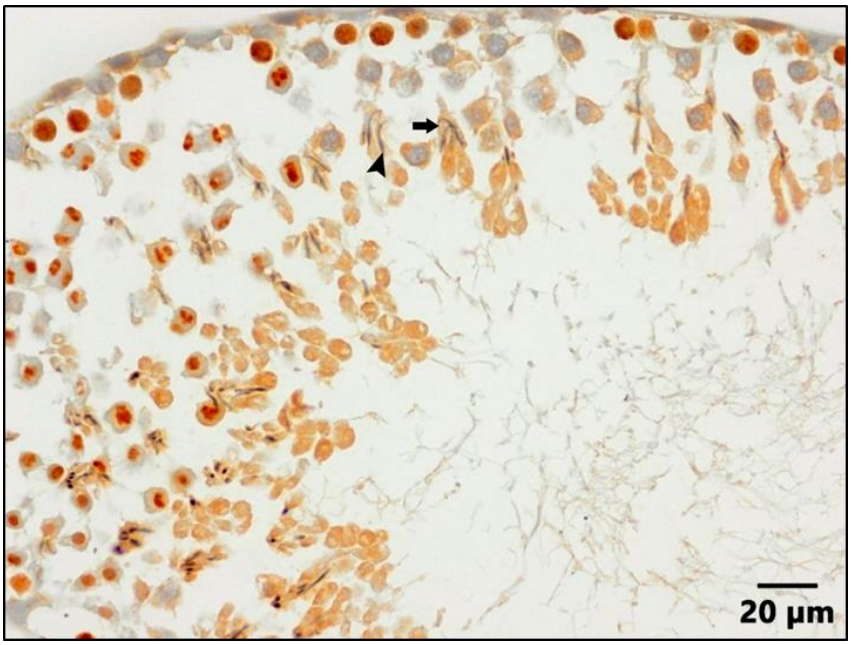

Figure 8. Tnp-1 expression in stage XIV seminiferous tubule section of an animal from the CIS-5 experimental group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

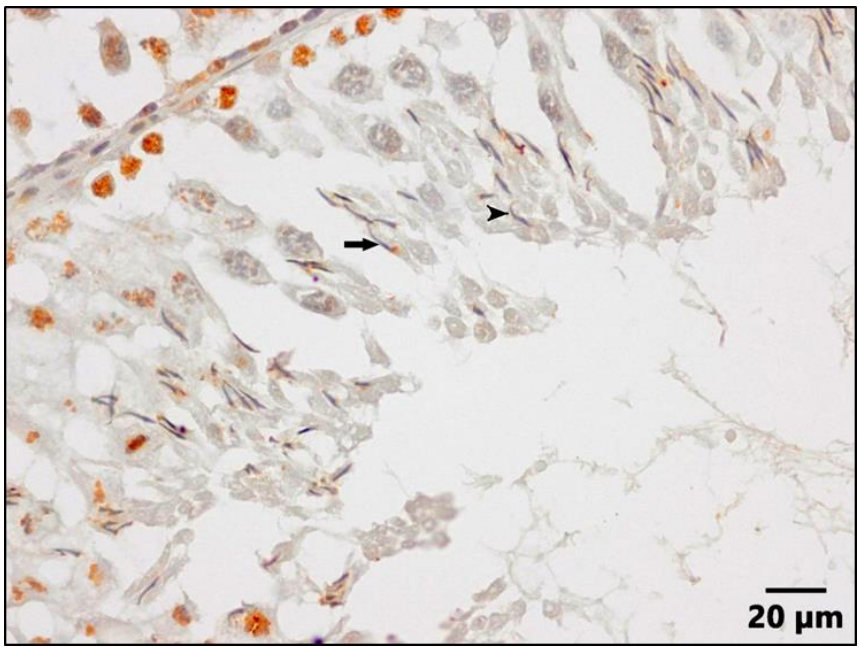

Figure 9. Tnp-1 expression in stage XIV seminiferous tubule section of an animal from the control group. Positive (arrow) and negative elongated spermatids (arrowhead) are seen. Streptavidin Biotin Method, Bar: 20 µm.

Apoptotic Cell Count And TUNEL Positive Tubulus Rate

Since degeneration in the tubules was observed in the experiment group of CIS CMN-15, this group was not taken into consideration. The number of apoptotic cells per tubulus and the percentage of TUNEL positive tubules according to the groups are given in Table 4. When the data are examined; the number of apoptotic cells increased in the cisplatin and cisplatin + curcumin groups compared to the control groups (Figure

10-D) and the groups given only curcumin. It was noticed that this increase was quite evident in the CIS CMN-10 (Figure 10-A), CIS-10 (Figure 10-B) and CIS-15 (Figure 10-C) groups. % TUNEL positive tubulus ratio; it was found to be statistically significant in the CIS-5, CIS-10, and CIS-15 groups compared to the control groups, and in the CIS CMN-10 group compared to all groups (Table 4).

Table 4. Number of Apoptotic Cells Perseminiferous Tubule and % TUNEL Positive Tubulus Rate in Control and Experimental Groups

Group	Number of Apoptotic Cells Per Tubulus	% TUNEL Positive Tubulus Value
	$(\overline{x} \pm S_{\overline{x}})$	
CIS-5	2,73±0,29[a,j]	49,66±1,85[b]
CMN-5	0,8±0,1[b,k]	27±1,73[a]
CIS CMN-5	1,33±0,16[c,k]	32±3[a]
CIS-10	4,42±0,42[j]	50,66±13,42[b]
CMN-10	1,31±0,18[a,k]	35±2[a,b]
CIS CMN-10	20,41±0,78[d]	100[d]
CIS-15	5,2±0,38[e]	71,66±4,09[c]
CMN-15	0,6±0,1[f,k]	22,5±2,5[a]
Control Cg	0,4±0,07[g,k]	19,33±2,02[a]
Control Sp	0,4±0,05[h,k]	19±3[a]
Control Sp Cg	0,6±0,1[i,k]	23,5±0,5[a]

a,b,c,d,e,f,g,h,i,j,k: Groups with different letters in the same column are statistically significant ($p<0,05$). CIS 5: 5-day group given cisplatin, CMN 5: 5-day group given curcumin, CIS CMN-5: 5-day group given cisplatin and curcumin, CIS 10: 10-day group given cisplatin, CMN 10: 10-day group given curcumin, CIS CMN-10: 10-day group given cisplatin and curcumin, CIS 15: 15-day group given cisplatin, CMN 15: 15-day group given curcumin, Control Cg: Control group given corn oil, Control Sp: Control group given physiological saline, Control Sp Cg: Control group given saline and corn oil.

Electron Microcopic Findings

Electron microscopic examinations were carried out on samples taken 15 days after the experimental applications. When spermatogenetic process is observed in detail in thin sections belonging to the control group; A and B type spermatogonia (Figure 11-A) and Sertoli cells in the basal compartment, primary and secondary spermatocytes in the adluminal compartment, in the part close to the lumen; round, elongated (Figure 11-B), elongated spermatids and spermatozoa were observed. On the 15th day of cisplatin administration (CIS-15), thin sections taken from the testes of rats were compared with the control group; it was observed that the intercellular distance between basal compartment cells increased and intercellular separation occurred in most of the developmental stages. Increase in intercellular distance as a result of disruption in tight junction complexes between Sertoli cells and spermatogenetic cells was also observed among spermatocytes. In the same section, the increase in lipofuscin granules in the secondary spermatocyte was noted. In primary spermatocytes, the karyorrhexis stage of the necrosis, which is characterized

by the breakdown of the nucleus together with the increase in lipofuscin granules, was observed (Figure 11-C). Another spermatocyte with advanced necrosis was seen in the lumen together with the round spermatid (Figure 11-D).

When the cells in the CMN-15 experiment group were examined; increase in lipid droplets and swelling in mitochondria were noted belonging to spermatogonium in the basal compartment (Figure 11-E, F). An increase in intercellular distance between pale type A spermatogonium and spermatocytes and intense vacuolization was observed in a different region of the basal compartment (Figure 11 E). When the micrographs of the adluminal compartment were examined, it was noted that the primary spermatocyte cytoplasm increased vacuolization and the disruption of the tight junction regions (Figure 11-F). When thin sections belonging to Cisplatin Curcumin-15 experiment group were examined (Figure 11 G, H), a more striking degeneration was encountered. In addition to intercellular separations in the cells of the basal compartment, the formation of a multilamellar body in the cytoplasm, degeneration with loss of cristae in the mitochondrium, autophagic vacuoles, vacuolization and excessive increase in lipid droplets were noted.

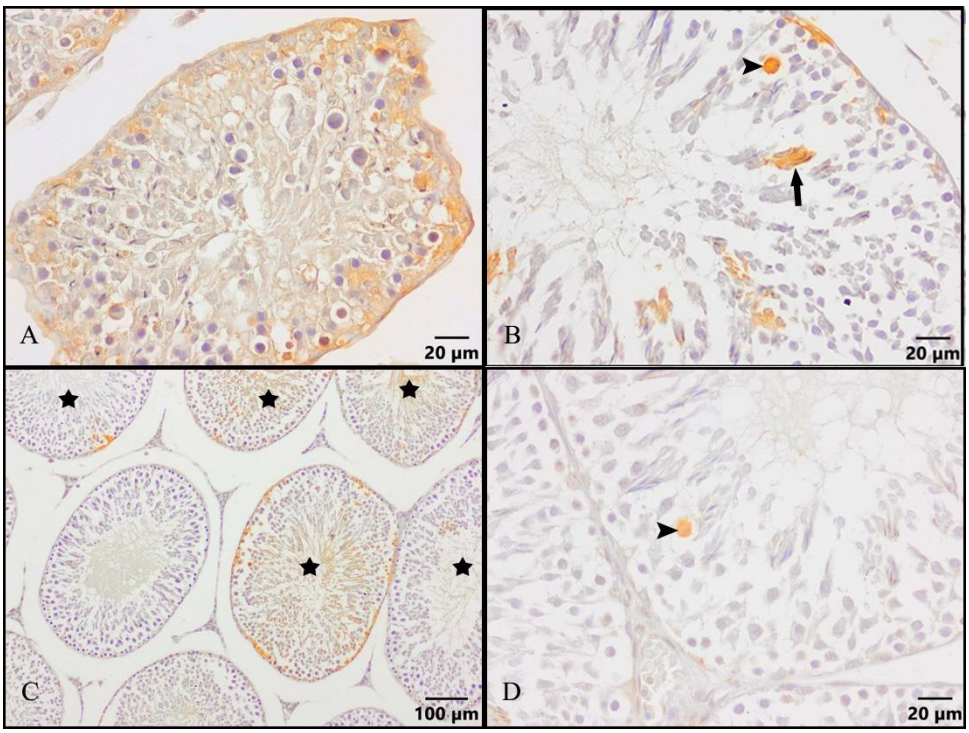

Figure 10. Apoptotic cells in testicular sections belonging to experimental groups and control groups. Positivity is observed in degenerated spermatogenetic cells in the testis section of an animal from the CIS CMN-10 experimental group (A). Positive spermatocytes (arrowhead) and positive elongated spermatids (arrow) are seen in the testis section of an animal from the CIS-10 experimental group (B). TUNEL positive seminiferous tubules (asterisks) from an animal from the CIS-15 experimental group (C) and positive spermatocytes (arrowhead) from an animal from the Control Sp group (D) are seen. TUNEL method, Bar: 20 µm (A,B,D); 100 µm (C).

When thin sections of the adluminal compartment are examined; again, as a result of increased vacuolization and disruption in intercellular junction complexes, intercellular separation was intense. There were necrotic cells in the same area

(Figure 11 G). Along with the formation of multilamellar bodies in round spermatids, degeneration with loss of cristae in mitochondrium and hypertrophy in the endoplasmic reticulum, the increase in lipid droplets and apoptotic bodies containing organelles surrounded by membranes were noted (Figure 11-H).

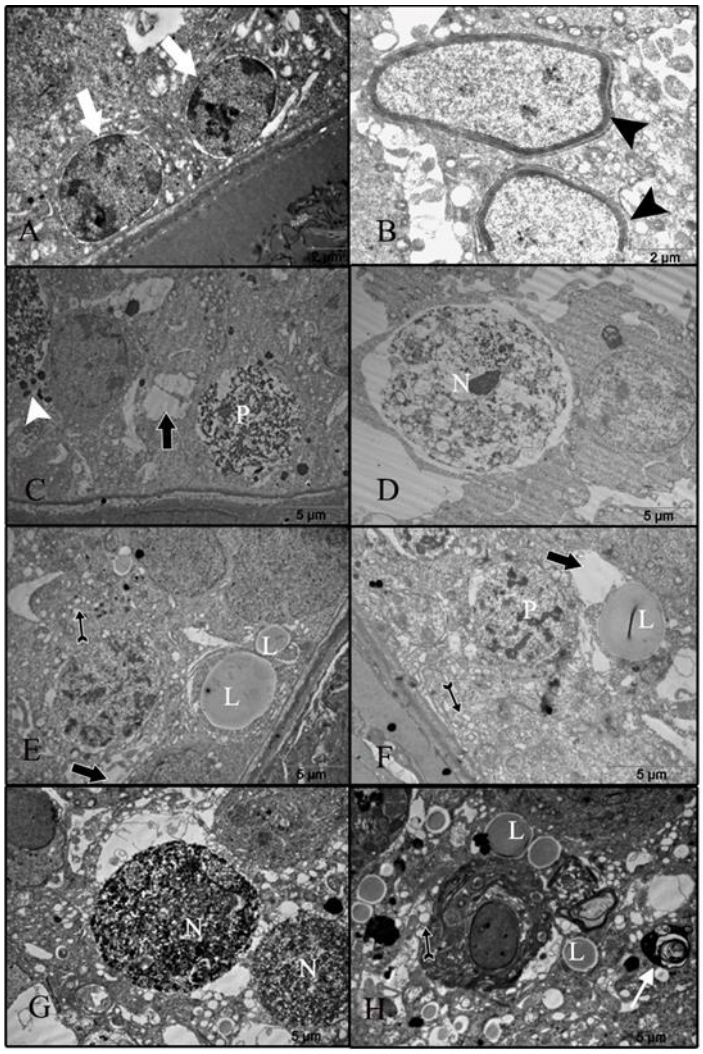

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Figure 11. Electron micrograph of the testis belonging to the Control Sp group (A-B) shows type B spermatogonium (white arrow), and elongated spermatid (black arrowhead) belonging to the stage X. Bar: 2 µm.

Separation between spermatogenetic cells (black arrow), lipofuscin granules (white arrowhead) in primary and secondary spermatocytes, necrotic primary spermatocytes (P), and necrotic cells (N) in the adluminal compartment are seen in the testicular electron micrograph (C-D) of the CIS-15 experimental group. Bar: 5 µm. In the testis electron micrograph of the CMN-15 experiment group (E-F), lipid granules (L, 11-E) in spermatogonium, separation between spermatogonium and primary spermatocytes (black arrow, 11-E), vacuolization in primary spermatocyte cytoplasm (double headed arrow, 11-E) lipid droplets (L, 11-F), primary spermatocyte (P), disruption in junction complexes (black arrow, 11-F), vacuolization (double headed arrow, 11-F) are seen. Bar: 5 µm. In the electron micrograph of the testis (G-H) of the CIS CMN-15 experimental

group; Necrotic cells (N) multilamellar body (white thin arrow), lipid droplets (L), vacuolization (double headed arrow) are seen. Bar: 5 µm.

Atessahin et al. on 2006 applied lycopene for 10 days before giving cisplatin or 5 days after cisplatin in their study. In all experimental groups, both right and left testicular weights were found to be significantly lower compared to the control group. Seminiferous tubule diameters were found to be significantly lower in the group given only cisplatin compared to the control group. In our study, on the contrary to this study, it was observed that the weight of the testes taken on the 5th day of the application (CIS-5, CIS CMN-5) increased compared to the controls (Table 1). On the 10th and 15th days of the application (CIS CMN-10 and CIS KMN-15) it was observed that testicular weights decreased compared to controls. According to the seminiferous tubulus diameter measurement results of our study, it was observed that the tubulus diameters were narrowed in the experimental groups CIS-10, CIS CMN-10 and CIS CMN-15, and this narrowing was statistically significant (Table 1, Figure 2).

In a study, the antiapoptotic and antioxidant effects of eugenol on cisplatin-induced testicular toxicity were investigated.(Ekinci Akdemir et al. 2019) In this study, sprague Dawley rats were injected with a single dose of 15 mg / kg cisplatin ip and the animals were sacrificed after 72 hours. In the study, a significant decrease in total testicular weight was found in the cisplatin group. When compared to our study, there is a difference in this study in terms of both the amount of dose given and the day the animals were sacrificed. As a matter of fact, in our study, an increase in testicular weights rather than a decrease was found on the 5th day of cisplatin administration compared to controls (Table 1).

Park et al. (2016) (Park et al. 2016), in their study on 253J-Bv (p53 wild-type) and T24 (p53 mutant) human bladder cancer cell lines, showed that cisplatin combined with curcumin treatment targeted the extracellular regulated kinase (ERK) signal, and noted cisplatin treatment increased rate of apoptosis in 253J-Bv and T24 cells. Although this study conducted with cancer cell lines was consistent with the TUNEL findings in the increased apoptosis rates of the cisplatin-curcumin experimental group of our study, its effect on normal cells was not shown in this study. From this point of view, the findings of our study are important (Table 4, Figure 10).

The study by Boroja et al. in 2018 (Boroja et al. 2018) looked at Bax and Bcl-2 expressions to investigate the regulatory effects of the herb Satureja hortensis known as savory against oxidative damage induced by cisplatin in kidney and testicles. The results of this study showed that antioxidant treatment has protective effects against cisplatin-induced apoptosis, which is evidenced by the increase in Bcl-2 gene expression. In the results of our study, although apoptosis increased significantly in the CIS-10 and CIS-15 groups, administration of curcumin further exacerbated the increase in apoptosis (Table 4, Figure 10) . Again, in the histopathological findings of the same study, it was stated that there was mild degeneration in seminiferous tubules and Sertoli cells in testicular tissue samples of the groups given antioxidants with cisplatin. When compared with the histopathological results in our study, the presence of degeneration in the group given antioxidant with cisplatin was similar (Table 2, Figure 1). Although degeneration was more severe in the experimental groups CIS CMN-10 and CIS CMN-15 in our study, in the study of Boroja et al. (2018), it is thought that giving antioxidant before cisplatin injection has a protective effect.

Mercantepe et al. (2018) (Mercantepe et al. 2018) performed a study to examine the benefits of antioxidants such as amifostine, curcumin and caffeic acid phenyl ester (CAPE) on cisplatin testicular damage. In this study 100 mg / kg curcumin

was administered once 24 hours before cisplatin injection and tissue samples were taken at the end of 7 days. In the light microscopic results of this study, it was found that in the cisplatin group, there was widespread oedema in the testicular tissue due to the loss of germinal epithelial cells in the seminiferous tubule, the presence of spermatogonia with atypical nuclei containing dense chromatin and degeneration of the connection between spermatocytes, the decreased number of spermatozoa and spermatids less than the control group, degeneration of basal lamina of seminiferous tubules and intense hyalinization in the interstitial space. In the cisplatin + curcumin group, it was found that there was a small amount of vacuolization between the seminiferous tubule cells, spermatogonium and spermatids were normal, spermatozoa were more numerous than the cisplatin group, and hyalinization was less in the interstitial space. In our study, while degenerative changes were not very evident in the CIS-5 group, these degenerative changes were more striking in the CIS-15 group. However, contrary to the findings of Mercantepe et al. (2018), in our study, cisplatin findings were exacerbated in the experimental groups of CIS KMN-10 and CIS CMN-15 (Table 2, Figure 1). In our study intercellular separations due to cell loss, cell debris in lumen, spermatogenetic arrest, apoptotic cells, dense appearance in spermatocyte chromatin, irregular shape of the tunica albuginea layer defining the tubular boundaries, and testicular atrophy due to tubular shrinkage were observed. Again, in the study of Mercantepe et al. (2018), according to the findings of caspase-3 immunohistochemistry, which is an apoptosis marker, in the group given cisplatin immunpositive cells were observed to be increased, especially in A and B type spermatogonia, primary spermatocytes and spermatids. It has been reported that in the cisplatin + curcumin group, immunpositive cells decreased in Sertoli cells and spermatids. In the results of our study, although apoptosis increased significantly in the CIS-10 and CIS-15 groups, administration of curcumin further exacerbated the increase in apoptosis (Table 4, Figure 10). We think that the reason for the differences in the cisplatin + curcumin group is that we used curcumin at twice the dose in our study and the application interval and time were different.

In the study of Ekinci Akdemir et al. (2019) (Ekinci Akdemir et al. 2019) investigating the antiapoptotic and antioxidant effects of eugenol on cisplatin-induced testicular toxicity Sprague Dawley rats were injected ip with a single dose of 15 mg / kg cisplatin and the animals were sacrificed after 72 hours. In the histopathological findings of this study, degeneration and necrosis in spermatogonia, a small number of spermatozoa in the lumen, oedema in the interstitial spaces, vascular dilatation and hyperemia were detected in the cisplatin group. These findings are seem to be parallel to our light microscopic findings. Although there was a difference in dose and duration in this study, according to the Johnsen criteria evaluation we made in our study, although it was more severe in the groups given curcumin with cisplatin, all cisplatin groups were found to be significantly different from control groups (Table 2). In the same study, when the expression of caspase-3 was compared between the groups, the expression in the cytoplasm of spermatogonium and spermatocyte was found to be more severe in the cisplatin group. While no caspase-3 expression was observed in the group given only eugenol, moderate expression was observed in the group administered with eugenol with cisplatin compared to the group given cisplatin. In our study, it was found that the rate of TUNEL positive tubules increased significantly in the groups given curcumin with cisplatin and cisplatin compared to the control groups.

Coadministration of the chemotherapeutic cocktail bleomycin, etoposide and cisplatin (BEP) has increased the 5-yr survival rate of testiscancer patients to over 90%. (Einhorn and Foster 2006; Huddart and Birtle 2005; Kopp et al. 2006) However,

it has been shown that BEP has permanent effects on the proteins in the sperm head that participate in chromatin formation. In the study conducted by O'Flaherty et al. in 2012 (O'Flaherty et al. 2012), it has been shown that BEP, which causes significant DNA damage and a decrease in chromatin condensation in patients treated with BEP until the 24th month after treatment, has a permanent effect on sperm chromatin quality. With the significant increase in the number of somatic histones in Brown Norway Rats treated with BEP in the studies of Maselli et al. in 2012 and 2013 (Maselli et al. 2012; Maselli et al. 2013), it has been suggested that the expression of PRM 1, the only known protamine (PRM) in rats, is decreased, and therefore histone withdrawal in germ cells is problematic.

Bagheri-Sereshki et al in their study on rats in 2016 (Bagheri-Sereshki et al. 2016); to allow the transcription of genes involved in the remodeling of chromatin in pachyten spermatocytes after BEP treatment in western Blot analysis showing not destabilization of nucleosomes, have shown expression of H3K9m (monomethylation at 9th lysis of H3 histone) was significantly increased and expression of tH2B (or Hist1h2ba) was significantly decreased. Moreover, BEP treatment altered the H4K8ac expression, which showed problematic histone withdrawal and caused loose chromatin structure in mature spermatozoa. It is known that less condensed sperm chromatin has negative effects on male fertility by making changes in the sperm epigenome. Immunohistochemical findings showing histone modifications in the same study confirm Western Blot analysis. BEP treatment significantly decreased the immunofluorescence density of H4K8ac in round spermatids of stage I-III, while significantly increasing it in round spermatids of stage IV-VI. These results showed that the changes induced by BEP treatment on histone markers involved in chromatin remodeling are cell and stage specific. The immunohistochemical results of this study support our immunohistochemical findings in our 5-day (CIS-5) experimental group treated with cisplatin. According to our results, TP1 protein immunopositivity was found to be cell and stage specific, and in all three stages examined in CIS-5 experimental groups, elongated spermatids were significantly higher than controls (Table 3, Figure 3-9). Although there are no studies on TP protein related chemotherapeutic agents, Western blot analysis is recommended for future studies. When the immunohistochemical findings of our study are considered in this perspective, it is a first study.

On the other hand, reactive oxygen species is known to cause oncogenic mutations and activate oncogenic pathways. However, it has been reported that treatment with antioxidants initiates some cancers or, on the contrary, suppresses the progression of some cancers.(Gorrini et al. 2013; Lewis et al. 2014; Fan et al. 2014; Ye et al. 2014; Chandel and Tuveson 2014) Similarly; although some studies have shown that antioxidants or antioxidant enzymes suppress the onset of cancer (Gao et al. 2007; Teoh-Fitzgerald et al. 2014; Glasauer et al. 2014); it has also been reported in some studies to stimulate the onset of cancer.(Harris et al. 2015; Sayin et al. 2014; DeNicola et al. 2011; Glasauer and Chandel 2014). Our study showed that the antioxidant given together with cisplatin further aggravates the histopathological, immunohistochemical and electron microscopic findings in the testis. Our study is not a cancer study, but it has shown that the use of antioxidants in combination with chemotherapeutics may be destructive.

According to the electron microscopic results of Mercantepe et al. (2018) (Mercantepe et al. 2018), it was reported that in the cisplatin group, there was degeneration in the basal lamina, apoptotic spermatogonium and spermatocytes, fragmentation in the spermatogonium and spermatid nuclei, condensation in the

spermatogonium nuclei and spermatids, vacuolization in mitochondria of spermatogonia and spermatids. In the cisplatin + curcumin group, it was shown that the basal lamina, spermatid and spermatocytes and mitochondria containing dense matrix had a normal structure. Although we obtained similar findings in the cisplatin-administered group in our study, it was observed that the adverse effects were exacerbated in the cisplatin + curcumin groups (Figure 11). Namely, the formation of multilamellar bodies in the cytoplasm, degeneration seen with the loss of cristae in mitochondrium, autophagic vacuoles, apoptotic bodies, excessive increase in vacuolization and lipid droplets have been noted.

In the study conducted to Aydıner et al. (1995) (Aydıner et al. 1995) administered 5 mg / kg cisplatin ip as ip, and after 3, 12, 21 and 28 days, electron microscopy findings showed SER dilatations on the days 3 and 12, disruption in mitochondria structure, disruption in the connection between Sertoli-germinal cells and germinal cells; it was observed that these deteriorations started to improve in the experimental group of day 21 and returned to normal in the experimental group of day 28. It was reported that the increase in lipid and lipofuscin granules started to improve in the experimental group of day 28. The electron microscopic findings of this study support our electron microscopic findings in our 15-day (CIS-15) experimental group treated with cisplatin (Figure 11). In our study, when compared with the control group on day 15 of cisplatin administration, it was observed that the intercellular distance between basal compartment cells increased and intercellular separation occurred in most of the developmental stages, especially the tight junction complexes between Sertoli cells and spermatogenetic serial cells were disrupted. Vacuolization within the cytoplasmic extensions of Sertoli cells extending between the cells in the adluminal compartment was noted, and an increase in lipofuscin granules in the cytoplasm was observed in these cells. Increase in intercellular distance as a result of disruption in tight junction complexes between Sertoli cells and spermatogenetic cells was also observed among spermatocytes. With the increase in lipofuscin granules in secondary spermatocytes, loss of cristae in the mitochondria lined up around the plasma membrane in round spermatids was noted.

The expression of Tnp-1 was revealed for the first time in this study in order to see the negative effects of a cancer drug, Cisplatin, in the testis and to test how the antioxidant curcumin would change these negative effects, especially in the nucleus. In our study, TNP-1 protein which can be detected in the stages of XII, XIII and XIV, expression was found to be statistically different in CIS-5 experimental group. It was also observed that this antibody decreased in the SIS-10 experimental group for all three stages and reached a value close to the control in the SIS-15 experimental group. These findings suggest that events during DNA packaging may be affected by cisplatin and that loose packaging of chromatin may lead to infertility. One of the strengths of this study is that we obtained histopathological, immunohistochemical and apoptotic findings 5, 10 and 15 days after the application of the cancer drug. In other words, the findings include significant changes on these different days, and these changes have guided us in the interpretation of the findings. We found dramatic changes histopathologically especially in the CIS KMN-10 and CIS KMN-15 groups. These findings show us that curcumin treatment applied together with cisplatin increases the damage in cells. In addition, the fact that we confirmed our findings with electron microscopic findings shows another strength of this study. On the other hand, if we had the opportunity to perform molecular techniques on the samples we took and we could support our findings with these tests, our study would be even stronger.

CONCLUSION

Cisplatin, an antineoplastic agent used especially in the treatment of testicular cancers, may cause testicular atrophy by causing narrowing in seminiferous tubule diameters depending on the dose and duration of administration. Moreover molecular technique analysis is recommended, which we believe will provide more objective results for future DNA packaging studies. In future studies using tumor models, the use of curcumin with cisplatin and its effects on cancer suppression by dragging these cells into apoptosis can be evaluated. Both light and electron microscopic findings showed that in time dependent manner there was arrest in spermatogenesis and because of germ cell losses extensive morphological degeneration in the groups given curcumin with cisplatin. As stated in the discussion, in previous studies, curcumin was given before cisplatin injection and it was found to have a protective effect. Although curcumin was given together with cisplatin injection in this study, it was observed that negative effects increased dramatically rather than its protective effects depending on the duration. For this reason, physicians who use cisplatin for cancer treatment are advised not to use curcumin in diet together with Cisplatin in their patients because it exacerbates the side effects, namely the negative effects of the cancer drug. Studies on these negative results, which we mentioned in our findings, are limited. We believe that our study will shed light on future studies on DNA packaging and morphological changes, especially in cancer patients treated with cisplatin, and fill an important gap in the literature.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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