Researches on the protective effect of caffeic acid phenethyl ester on testicular damage caused by cisplatin

Abstract

Background/aim: Cisplatin (CP), a chemotherapeutic drug, causes damage to spermatogenic serial cells, Sertoli cells and Leydig cells in rat testicles. We aimed to investigate the protective effect of caffeic acid phenethyl ester (CAPE), one of the active ingredients of propolis, in eliminating CP - induced testicular damage.

Materials and methods: Group 1 (control group) was given physiological saline solution intraperitoneal (IP) throughout the experiment. Group 2 (CP group) was given a single dose of CP (7 mg / kg) IP on the 7th day. Group 3 (CP + CAPE group), CAPE (10 μmol / kg / day) IP for 12 days and single dose CP (7 mg / kg) IP on day 7. given as. Group 4 (CAPE group) was given CAPE (10 μmol / kg / day) IP for 12 days. On the 14th day of the experiment, the rats were decapitated under xylazine and ketamine anesthesia and their testicles were removed. The sections obtained from the testicles were stained with hematoxylin - eosin and histopathological damage was evaluated. Malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) enzymatic activities were measured in testicular tissue samples. Testosterone (TES) levels were measured in blood serum. Johnsen testicular biopsy score (JTBS) was used to evaluate testicular tubules. DNA damage was evaluated in sperm samples taken from the ductus epididymis by comet assay technique.

Results: In Group 2 given CP, the testicles were severely damaged. It was observed that histological damage was reduced in testes by giving CAPE in Group 3. Besides, according to JTBS, the value was significantly higher in testicular tubules ($p < 0.05$). Moreover, the MDA level was decreased in Group 3. But, SOD, CAT and TES levels

were increased in Group 3. DNA damage also decreased significantly in Group 3 compared to Group 2 ($p < 0.05$).

Conclusion: As a result, CAPE may be protective against damage caused by CP in the testicles.

Key words: Caffeic acid phenethyl ester, cisplatin, comet assay, rat, testes

1. Introduction

Chemotherapy drugs are used to the treatment of various types of cancers. Their toxicities and minor risks are often overlooked because of their potential usefulness in treatment. In particular, testicular cells are selected as targets by chemotherapeutic agents as they enter various mitotic, meiotic and morphological processes. Therefore, they are easily damaged [1]. Cis - diamminedichloroplatinum (II) or cisplatin (CP), a highly effective antineoplastic DNA alkylating agent; It is used to treat a wide variety of solid tumors such as testicles, ovaries, breast, lung, bladder, head and neck [2]. However, the use of the drug is limited due to side effects such as testicular toxicity, cachexia and testicular damage [2,3]. When CP is administered at a high cumulative dose, it can develop permanent azoospermia and then infertility in patients [3]. CP interacts with DNA to create cross-link and intra-chain cross-links. Since the DNA modified by CP cannot be renewed enough, DNA damage initiates apoptosis [4].

Caffeic acid phenethyl ester (CAPE) is one of the active ingredients of the sharp and fragrant propolis substance found in the extract collected by bees from plants. CAPE has been used as a folk remedy for many years [5]. Antimicrobial, anti-inflammatory, immunomodulatory, antimutagenic, antioxidant and anticarcinogenic effects of propolis have been demonstrated by various studies. CAPE, one of the active ingredients of propolis, has an important place in these beneficial effects [6,7]. Although CAPE is a

pharmacologically safe compound, it also reduces lipid peroxidation and stimulates antioxidant enzyme activity [8].

In this study, we evaluated the effects of CAPE on testicular damage, DNA fragmentation and antioxidant capacity mechanisms in CP - induced testes injury.

2. Material and methods

2.1. Animals and drug administration

The study protocol was accepted by the Erciyes University's Experimental Animal and Local Ethics Committee (decision no: 15 / 59). This study was supported by the Erciyes University Scientific Research Projects' Unit with the number of TYL – 2015 - 5948. 38 male adult *Wistar albino* rats weighing between 150 and 220 g 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.

The animals were randomly divided into four groups. Group 1 was given physiological saline solution (1 ml / kg / day) intraperitoneally (IP) throughout the experiment. Group 2 (CP group) was given a single dose of CP (Koçak®) (7 mg / kg) IP on the 7th day of the experiment. The dose of CP was selected according to previous study that demonstrated significant testicular toxicity in rats [9]. Group 3 (CP + CAPE group) was given CAPE (Sigma®) (10 μmol / kg / day) IP for 12 days and single dose CP (7 mg / kg) IP on the 7th day of the experiment. The dose of CAPE was selected based on the results of recent studies where the antioxidant and anti - inflammatory action of this agent was apparent [10,11]. Group 4 (CAPE group) was given as CAPE (10 μmol / kg / day) IP for 12 days. At the end of the study rats were decapitated after intraperitoneal ketamine (75 mg / kg) xylazine (10 mg / kg) anesthesia and the testis tissues were taken

out rapidly. The sections obtained from the testicles were stained with hematoxylin-eosin (HE) and histological damage was evaluated. Malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) enzymatic activities were measured in testicular tissue samples. Testosterone (TES) levels were measured in blood serum. Johnsen testicular biopsy score (JTBS) was used to evaluate testicular tubules. DNA damage was evaluated in sperm samples taken from the ductus epididymis by comet assay technique.

2.2. 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. HE paints were purchased from Nanotek Lab. The slides were then examined under an Olympus BX51 microscope.

2.3. Johnsen testicular biopsy score

According to this scoring, after any event causing damage, the distribution of the cells in the testicle tubules disappears progressively, following a certain order. JTBS was used to assess the degree of this damage to the tubules [12]. The results of histological examinations were evaluated by two specialist histologists at the Department of Histology and Embryology, Erciyes University Medical School. Evaluation was made by examining 20 different tubules from 10 different preparations randomly selected from each group. For each group, 200 tubules were evaluated separately and the average JTBS was calculated. SPSS package program was used for statistical comparisons

between groups. The data obtained were processed in a form prepared previously (Table 1).

2.4. Biochemical analysis

Biochemical analysis was performed according to the manufacturer's Enzyme - Linked Immunosorbent Assay (ELISA) kit procedures. The study was carried out in the Department of Histology and Embryology Laboratory of Erciyes University Medical Faculty, using commercial kits of YH Biosearch Labratory, obtained from Molgen Biotechnology Lab. [rat malondialdehyde (MDA) ELISA kit cat no: YHB0708Ra, rat super oxidase dismutase (SOD) ELISA Kit Cat No: YHB1021Ra, rat catalase (CAT) ELISA kit cat no: YHB0207Ra, rat testosterone cat. no**:** 201 - 11 - 5126, Shanghai Sunred Biological Technology]. The results of tissue samples were given as nmol/mg SOD for MDA and ng/mg protein for CAT and the results of serum samples were given as nmol / ml for TES.

2.5. Comet assay technique

Diluted sperm samples extracted from epididymis were centrifuged at 300 g for 10 min at 4°C. The supernatant was removed and the remaining sperm cells were washed with Ca^{+2} and Mg^{+2} free PBS. Sperms with fragmented DNA were determined using the single-cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. The images of 100 randomly chosen nuclei from sperm sample of each animal were visually analyzed and sperms with fragmented DNA were counted. Observations were made at a magnification of × 400 using a fluorescent microscope (Olympus, Tokyo, Japan). The damage was determined from the broken DNA tail that migrated from the sperm head and caused comet. Cells with tails were evaluated as damaged [13].

2.6. Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 21.0). The Kolmogorov–Smirnov test was used to identify normal distribution of the data. In case of normal distribution, quantitative variables were compared using One-way analysis of variance (ANOVA) and post hoc Tukey test. Descriptive statistics were shown as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Light microscopic examination

In Group 1, the testicles appear to have normal histological features. HE staining images of the control group are shown in Figure 1A. In Group 2, there are intercellular spaces and local cell loss in areas near the lumen. The fibromuscular area was observed to be impaired. In some areas, basement membrane losses were observed. In the sections, deterioration in the basement membranes of tubule seminar counters was observed. However, the nuclei of the cells in the lumen were evident, but they had no tail structures. Leydig cells in the interstitial space were partially damaged. Interstitial oedema and cellular spaces were observed in the cell stages that should have belonged to Spermatid serial cells. HE staining images of the CP group are shown in Figure 1B. Tubules were observed to be close to normal in Group 3. There were morphological signs of improvement in Leydig cells in the interstitial space. In the sections, seminiferous tubules were close to normal. Spermatogenic serial cells were significantly close to normal. The basement membrane was properly observed. HE staining images of the CP + CAPE group are shown in Figure 1C. In Group 4, testicular histology was seen close to the control group. HE staining images of the control group are shown in

Figure 1D. In addition, HE staining images of all groups are shown in Figure 2. Figure 2A shows the normal seminiferous tubule structure. Figure 2B shows impaired fibromuscular space, unspecific spermatogenic series cells, diffused basement membrane. The tubule structure close to normal is shown in Figure 2C and Figure 2D.

3.2. Johnsen testicular biopsy score results

The germinal epithelium in the seminiferous tubules was evaluated with JTBS. According to these results, biopsy score was found to be decreased in Group 2 compared to Group 1 and it was statistically significant ($p < 0.05$). The biopsy score in Group 3 increased numerically compared to Group 2, and this increase was statistically significant ($p < 0.05$). In Group 4, the biopsy score was decreased compared to Group 1. This decrease was statistically significant, although not as in Group 2 ($p < 0.05$). JTBS results are shown in Table 2.

3.3. Biochemical results

MDA level and CAT and SOD enzyme activities were evaluated in testicular tissue by ELISA technique. Also, TES level was evaluated in blood serum. When the analyzes for CAT were evaluated, Group 2 significantly decreased than Group 1. Besides, in Group 3 was higher than Group 2. These values were not statistically significant ($p > 0.05$). SOD level in Group 2 was lower than Group 1. But, SOD level in Group 3 was higher than Group 2. These values were not statistically significant ($p > 0.05$). When the analyzes for MDA level were evaluated, in Group 2 was found higher than Group 1 and Group 3 was lower than Group 2. These values were not statistically significant ($p > 0.05$). When the analyzes for TES were evaluated, in Group 2 was found to be lower than Group 1 and Group 3 was higher than Group 2. These values were not statistically

significant ($p > 0.05$). ELISA technique results, in which biochemical data are evaluated, are shown in Table 3.

3.4. Comet assay technique results

The microphotographic appearance of the fragmented DNA sperm and the Head DNA and Tail DNA numbers in all groups are presented in Table 4, respectively. According to the data obtained as a result of measuring the microphotographic images; decreasing the amount of head DNA or increasing the amount of tail DNA refers to DNA fragmentation in the cell. Only CP administration significantly increased sperm DNA fragmentation rate compared to Group 1 ($p < 0.05$). Comet assay results of the Group 1 and Group 2 are shown in Figure 3A - 3B. Sperm DNA fragmentation rate decreased significantly ($p < 0.05$) in Group 3 compared to Group 2. Comet assay results of the Group 2 and Group 3 are shown in Figure 3B – 3C. Although CAPE application alone increased the rate of sperm DNA fragmentation compared to the control group, this increase was less than that of Group 2 ($p < 0.05$). Comet assay results of the Group 1 and Group 2 are shown in Figure 3D.

4. Discussion

Cisplatin (CP) is a drug used to treat some types of cancer in chemotherapy [14]. CP is an organic platinum-derived drug that is unlike any other antineoplastic drug in its structure. Testicular, ovarian, bladder carcinomas in particular; It is sensitive to CP based chemotherapy [1]. CP, a chemotherapeutic drug, causes a number of side effects, including cachexia and testicular damage [3]. Besides side effects such as nephrotoxicity, neurotoxicity, gastrointestinal irritability, it has been reported that it causes azoospermia, oligospermia and infertility [1]. CP therapy may develop

permanent azoospermia and subsequently infertility in patients receiving a high cumulative dose [3].

Testicular damage due to CP occurs directly on spermatogenic cells and Sertoli cells. It also causes dysfunction in Leydig cells. It was reported that sperm production, seminiferous tubule diameter, and intratesticular testosterone decreased and TUNEL positive cells increased in seminiferous tubules in CP - treated rats [14]. The most important point in cancer chemotherapy with CP is the protection against side effects [15].

Soni KK et al. when they apply CP; in testicular tissue have been reported abnormal histopathological lesions in germinal cells, Sertoli cells and Leydig cells [16]. Soni KK et al. demonstrated in another study the degeneration and disorganization of germinal cells, Sertoli cells, and Leydig cells in the 10 mg / kg CP - treated group [17]. However, a decrease in spermatogenic cell density was reported. They showed that JTBS decreased significantly in the CP group. At the same time, there was a significant increase in MDA levels in the CP applied group. In our study, treatment with CP increased the level of MDA compared to the control group. Also, treatment with CP suppressed antioxidant activity by lowering CAT and SOD levels. Although biochemical parameters showed tissue damage, this damage was not statistically significant.

In our study, a single dose of CP (7 mg / kg) caused significant damage in rat testes. This damage was mostly in spermatogenic series cells, deterioration in the sequence of series cells, loss in basement membranes and in the areas near the lumen, (spleen-free) spermatozoon. According to histopathological evaluations, the degree of damage of primary and secondary spermatocytes was observed in the spermatogenic cell series.

Cells in the lumen of the seminiferous tubule were nucleated, but lost tail. Leydig cells in the interstitial area were also partially damaged. The order of the series of spermatogenic cells should not be differentiated. The results obtained in our study are compatible with the studies in which CP induced testicular damage was created. JTBS measurements suggest significant damage to the seminiferous tubules with CP.

Comet assay method is an important method used to determine the damage and level of DNA [18]. Sarıözkan S et al., showed the sperm DNA integrity induced by Taxans by comet assay technique in their study. In their study, Docetaxel, Paclitaxel, Docetaxel + Paclitaxel were demonstrated with the comet assay technique in which sperm DNA fragmentation was impaired compared to the control group [13]. In our study, we showed the CP - induced DNA damage in sperms using the comet assay technique.

Propolis is used by bees to cover holes and cracks in the hive, to repair and paste combs, to narrow the hive entrance and to facilitate defense. Propolis is a disinfectant material and provides disinfection of honeycomb eyes [19].

Caffeic acid phenethyl ester (CAPE) is one of the active ingredients of the sharp and fragrant propolis substance, which is extracted from plants by bees, and has been used in alternative medicine for many years [5]. The antimicrobial, anti-inflammatory, immunomodulatory, antimutagenic, antioxidant and anticarcinogenic effects of propolis have been demonstrated by various studies. Most of these effects are related to CAPE, one of the active substances of propolis [6,7]. CAPE has been shown to reduce lipid peroxidation and induce antioxidant enzyme activity, as well as a pharmacologically safe compound [8,20]. Also, CAPE has been shown to inhibit the growth of different types of transformed cells [5].

Ferreira RS et al. reported that they had applied 1, 5, 20, 25 and 50 μmol CAPE against CP-induced neurotoxicity in their model by cell culture and reported that the most effective concentration providing the highest protection against cell death was 10 μmol [10]. Abdel-Daim MM et al. reported that the combination of CAPE (10 μmol / kg) and betaine (250 mg / kg) against Abamectin-induced hepatoxicity and nephrotoxicity, which are commonly used as plant protection and insecticides, have a protective effect [11]. Ayla S et al. showed that in sperm samples incubated with CAPE, DNA damage was decreased with low chromatin condensation compared to samples not incubated with CAPE, and at the same time, incubation with CAPE reduced MDA levels [21]. Yilmaz HR et al. investigated the anticlastogenic effect of CAPE on CP-induced chromosomal defects in rat bone marrow cells [22]. CP (5 mg / kg) was applied and CAPE was applied 24 h before (10 μmol / kg). Animals treated with single-dose CP have been reported to exhibit both abnormal chromosomal error and abnormal metaphase in bone marrow cells. In rats treated with CP + CAPE, total chromosomal error number and total abnormal metaphase ratio were reported to be significantly lower ($p = 0.0001$). Armağan et al. aimed to treat the oxidative stress caused by the administration of methotrexate (IP, 20 mg / kg) by administering CAPE (IP, 10 μmol / kg) [23]. In their study, it showed that CAT activity in the methotrexate group was lower than that of the CAPE - treated group in the testis. It was also reported that CAT activity in the methotrexate group was lower than that of the CAPE - treated group. This study shows that oxidative stress induced by methotrexate can be reduced by applying CAPE [23].

In conclusion also, studies have shown that CAPE has a therapeutic effect on many tissues. In our study, we applied CAPE which is one of the active substances of propolis

to prevent the damage caused by CP in rat testis tissue. We observed improvement in seminiferous tubules in testicular tissues of rats treated with CAPE together with CP. An increase in the number of Leydig cells in the interstitial area and signs of morphological improvement were observed. Tissue sections were very close to normal and Sertoli cells showed significant improvement. Basal membrane and spermatogenic series cells were found to be close to normal. The JTBS measurement results showed that the application of CAPE together with CP would reduce the damage caused by CP. Comet assay findings showed that CP-induced DNA damage in spermatozoa was reduced by CAPE administration. Along with CAPE treatment in CP - induced testicular injury, the following were seen; The increase of SOD and CAT values and the increase of TES in blood serum may be evaluated as the symptoms of improvement, although not statistically significant. Different and higher doses of CAPE may make biochemical parameters more meaningful.

As a result, CP affects many organs and causes serious histopathological damage in the testis tissue. As to prevent this damage, it was shown that CAPE, which was given for protective purposes, could be protective and corrective in testis. We believe that different and higher doses of CAPE may improve the damage more.

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Table 1. Johnsen testicular tiopsy score

Score	**Histological Findings**	**Score**	**Histological Findings**
1	No cells in tubular section	6	There are few spermatids (5/tubule)
2	There are only Sertoli cells	7	There are too many spermatids without any sign of difference
3	Germ cells are just as spermatogonium	8	Late spermatids without mature spermatozoa
4	There are few spermatocytes (5/tubule)	9	There are few spermatozoa (5/tubule)
5	There are too many spermatocytes	10	Exact spermatogenesis is present with a large number of spermatozoa

Table 2. Johnsen testicular biopsy score results.

	JTBS
Group 1 (n = 8)	8.12 ± 0.647
Group 2 (n = 10)	4.979 ± 0.983*
Group 3 (n = 10)	6.483 ± 0.656**

Group 4 (n = 10)	5.521 ± 0.453
p value	< 0.05

Values are given as mean ± standard deviation (SD). $p < 0.05$

* Compared to Group 1 $p < 0.05$

** Compared to Group 2 $p < 0.05$

Table 3. Biochemical data of the experimental groups.

	CAT	**SOD**	**MDA**	**TES**
Group 1 (n = 8)	19.015 ± 8.404	1.222 ± 0.949	0.84 ± 0.206	23.06 ± 14.22
Group 2 (n = 10)	14.299 ± 6.645	0.978 ± 0.443	0.852 ± 0.12	18.56 ± 2.81
Group 3 (n = 10)	22.321 ± 10,01	1.526 ± 0.765	0.811 ± 0.09	26.99 ± 11.15
Group 4 (n = 10)	19.872 ± 16.041	1.615 ± 0.439	0.826 ± 0.087	26.85 ± 10.26
p value	> 0.05	> 0.05	> 0.05	> 0.05

Values are given as mean ± standard deviation (SD). $p < 0.05$

Table 4. Comet assay results.

	Head DNA	**Tail DNA**
Group 1 (n = 8)	98.969 ± 0.6	1.031 ± 0.6
Group 2 (n = 10)	79.28 ± 4.584 *	20.706 ± 4.579 *
Group 3 (n = 10)	88.383 ± 2.133 **	11.616 ± 2.133 **
Group 4 (n = 10)	84.733 ± 3.398	15.266 ± 3.398
p value	< 0.05	< 0.05

Head DNA and Tail DNA values were evaluated separately. Values are given as mean ± standard deviation (SD). $p < 0.05$

* Compared to Group 1 $p < 0.05$

** Compared to Group 2 $p < 0.05$

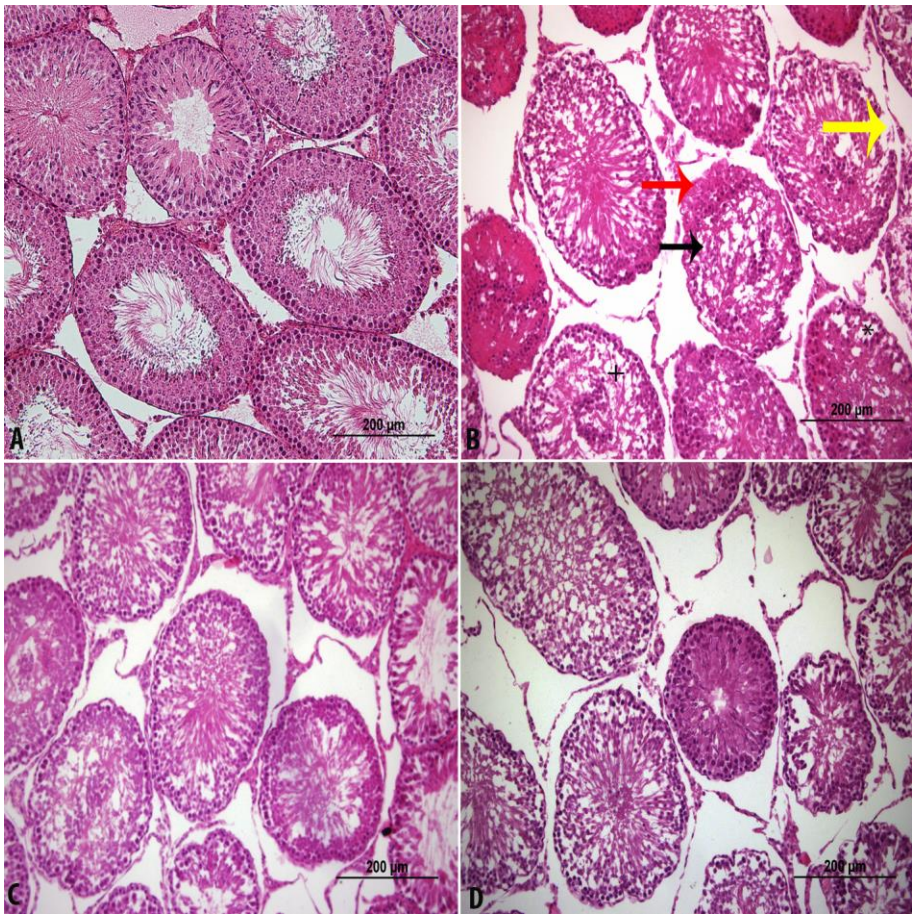

Figure 1. Control (A), CP (B), CP + CAPE (C) ve CAPE (D) (Hematoxylin - Eosin staining, × 200).

* Shows intratubular oedema

→ Shows unspecific spermatogenic series cells

→ Diffused basement membrane

→ Impaired fibromuscular space

+ Shows tailed spermatozoa in the lümen

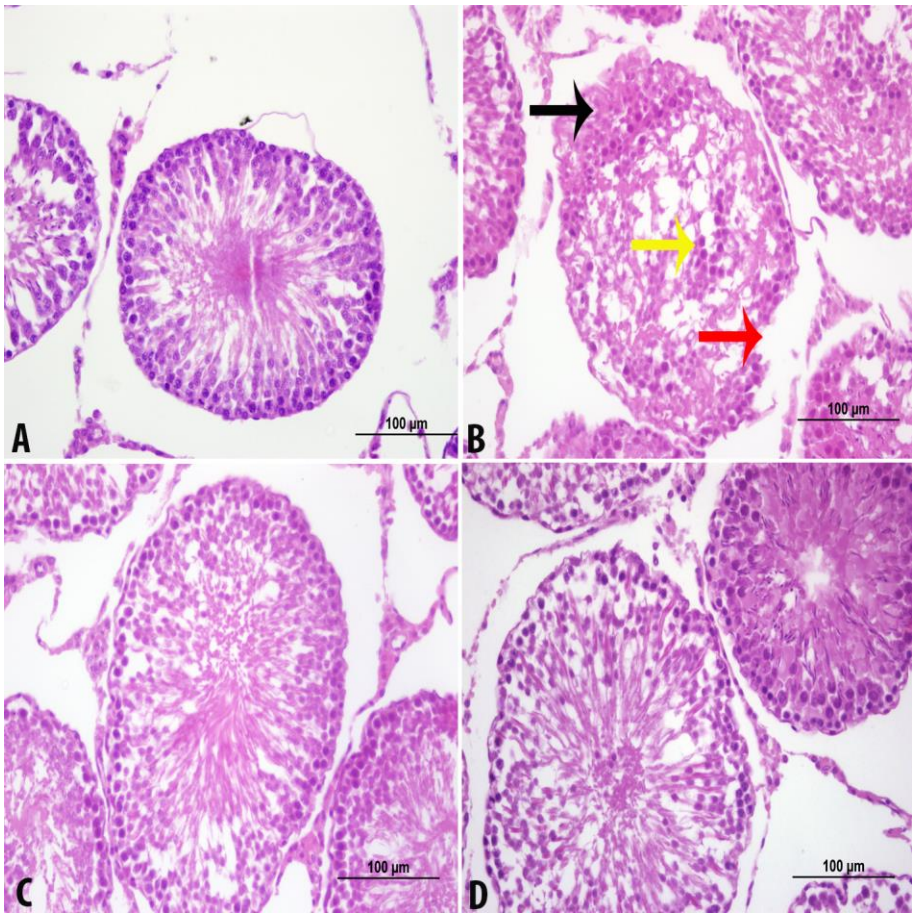

Figure 2. Control (A), CP (B), CP + CAPE (C) ve CAPE (D) (Hematoxylin - Eosin staining, × 400).

→ Impaired fibromuscular space

→ Shows unspecific spermatogenic series cells

→ Diffused basement membrane

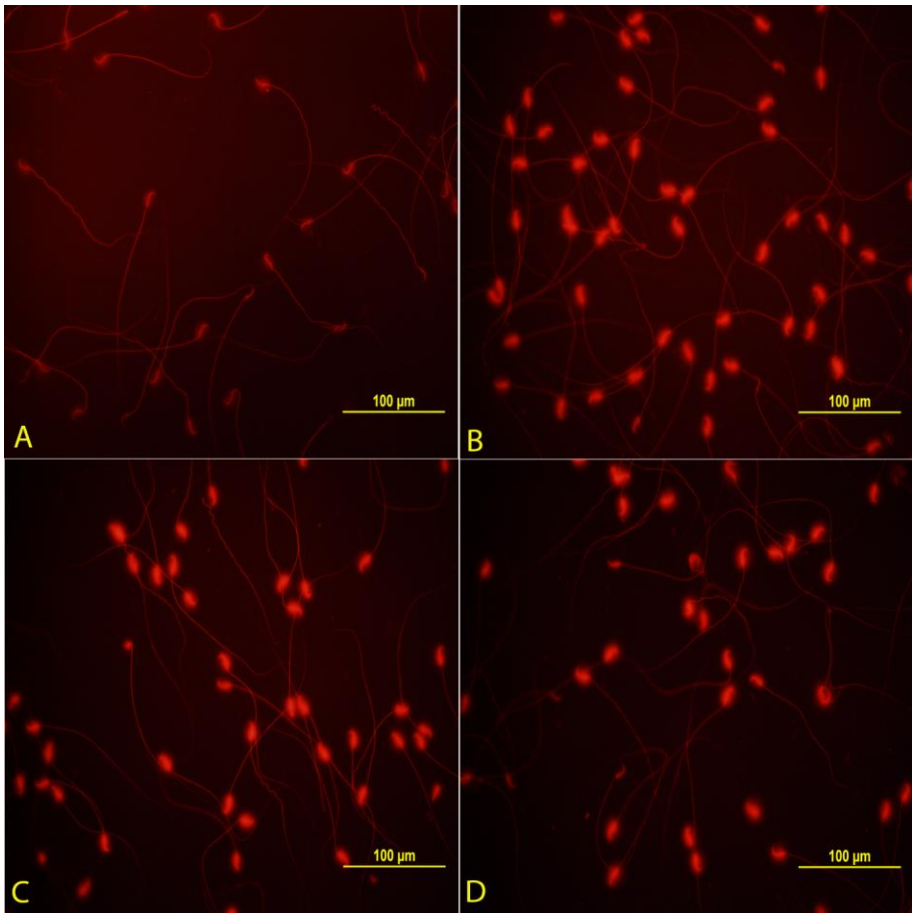

Figure 3. Control group (A); shows DNA undamaged sperm cells. CP (B), CP + CAPE (C) ve CAPE (D) groups; shows DNA damaged sperm cells. (Ethidium bromide staining, × 400).