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Investigation of the Effects of Cerium Nitrate and Platelet-Rich Plasma Treatments on Rat Testicular Tissue in the Recovery of the Stasis Zone

Seryum Nitrat ve Trombositten Zengin Plazma Tedavilerinin Yanıklardan Sonra Staz Bölgesinin İyileşmesinde Sıçan Testis Dokusu Üzerindeki Etkilerinin Araştırılması

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ABSTRACT

Post-burn trauma is common in daily life. Although a burn injury causes traumatic damage to the skin, it can also cause damage to many tissues and organs, including testicular tissue, as a result of systemic inflammatory reactions in the body. Cerium nitrate (CN) and Platelet-Rich-Plasma (PRP) are natural compounds having anti-inflammatory, anti-oxidant and anti-apoptotic properties. In this study, we aimed to investigate the efficacy of CN and PRP against the damage to testicular tissues after burns. Thermal damage was induced in the skin tissues of the rats on the first day of the study. Then, the rats in the CN group were kept in a 30-min bath of 0.04 M CN. The rats in the PRP group received 0.1 ml of PRP intradermal injections in the wound area. Spermatological examinations performed after burn revealed that abnormally shaped sperm counts increased and the integrity of the sperm membrane was impaired. In histopathological examinations, thinning of the tubular walls in testicular tissues was observed, as well as a decrease in spermatocyte numbers and severe degeneration and necrosis in the spermatocytes. It was also observed that the burn triggered inflammation in testicular tissues by increasing IL-1β, TNF-α and iNOS levels, caused DNA damage by increasing 8-OHdG levels, and furthermore caused apoptosis due to increased Caspase 3 expression in the testicular cells. It was determined that CN and PRP treatments reduced the number of abnormally shaped sperms after burn, maintained membrane integrity of sperms, and suppressed inflammation, oxidative stress and apoptosis in testicular tissues.

Keywords: Apoptosis, burn, cerium nitrate, inflammation, platelet-rich plasma

ÖZ

Yanık sonrası travma günlük yaşamda sıklıkla görülmektedir. Yanık yaralanması ciltte travmatik hasara neden olmakla birlikte, vücutta sistemik inflamatuar reaksiyonlar sonucu testis dokusu da dahil olmak üzere birçok doku ve organda da hasara neden olabilir. Seryum nitrat (SN) ve Trombositten Zengin Plazma (TZP), antiinflamatuar, antioksidan ve antiapoptotik özelliklere sahip doğal bileşiklerdir. Çalışmanın birinci gününde sıçanların cilt dokularında termal hasar oluşturuldu. Daha sonra CN grubundaki sıçanlar 0,04 M SN'li 30 dakikalık banyoda tutuldu. PRP grubundaki sıçanlara yara bölgesine 0,1 ml PRP intradermal enjeksiyonu yapıldı. Yanık sonrası yapılan spermatolojik incelemelerde anormal şekilli sperm sayısının arttığı ve sperm zarının bütünlüğünün bozulduğu görüldü. Histopatolojik incelemelerde testis dokularında tubüler duvarlarda incelme, spermatosit sayısında azalma ve spermatositlerde ileri derecede dejenerasyon ve nekroz gözlendi. Yanığın testis dokularında IL-1β, TNF-α ve iNOS düzeylerini artırarak inflamasyonu tetiklediği, 8-OHdG düzeylerini artırarak DNA hasarına neden olduğu ve ayrıca testis hücrelerinde Kaspaz 3 ekspresyonunun artmasına bağlı olarak apoptoza neden olduğu gözlendi. CN ve PRP tedavilerinin yanık sonrası anormal şekilli sperm sayısını azalttığı, spermlerin membran bütünlüğünü koruduğu, testis dokularında inflamasyonu, oksidatif stresi ve apoptozu baskıladığı belirlenmiştir.

Anahtar Kelimeler: Apoptozis, inflamasyon, seryum nitrat, trombositten zengin plazma, yanık

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INTRODUCTION

Burn injury is one of the most serious types of trauma that people are likely to experience at least once in their lifetime. At the present time, with the development of industrialization, many forms of trauma, particularly burns, are common in our daily lives. Burns usually occur by contact with a burning or hot material. As a result of the burn occurring in the tissues, due to the energy emission from the burning substance, coagulation necrosis develops within tissues and organs. The severity of the burn injury varies by the dose and severity of the flammable or combustible material, contact time of the tissue with the burning material, and the resistance of the exposed tissue.[1,2] Multisystem involvement of various tissues and organs, especially cardiovascular, respiratory and urinary systems, can be seen in very severe burns.[2] This is caused by oxidative stress and inflammatory reactions that develop in tissues and organs after burns.[3] Proinflammatory mediators cause severe inflammation and edema in the skin due to inflammatory reactions after burns. In addition to that, the inflammatory response that develops after a severe burn transforms into a systemic inflammatory response (SIRS3), affecting many tissues and organs.[4,5] Oxidative stress can cause permanent damage in the protein structures of cells, cell membranes, and lipid structure of cell DNA,[6] and at the same time, apoptosis can be observed in many cells.[7]

Although many tissues and organs in the body are severely affected by complications following burns, testicular tissues are affected even more because they are more sensitive to temperature changes. Testes are located in the scrotum sac at a temperature several degrees below the normal body temperature, which is very sensitive for spermatogenesis in testicular tissue. Oxidative stress and inflammatory reactions, which will occur due to the factors causing an increase in body temperature, such as burns, will also increase the temperature of the testes, leading to a serious decline in fertility and development of many diseases and damage, particularly cryptorchidism.[8-10] Burn wound treatment is still the subject of many experimental studies. Different treatment methods such as stem cells are widely used in studies, besides anticoagulants, anti-inflammatory, anti-thrombotic and anti-oxidant drugs. Essential active substances such as cerium nitrate (CN) and Platelet Rich Plasma (PRP) are used in these treatment methods.[5] CN, found very rare in nature, was determined to eliminate the immunosuppressive effect in the body after trauma, and to support the treatment positively by suppressing the inflammatory reaction after burn injury.[3] It was proven that PRP, obtained from the platelet-enriched plasma portion after centrifugation of blood, accelerates wound healing by affecting the release of growth hormones and anti-inflammatory cytokines in the body.[8] Nonetheless, PRP has been widely used in many treatment methods in recent years, especially in surgery and dermatology. Recent studies also revealed that PRP was very effective in the treatment of burn wounds.[10]

Various methods were used in this study, with a view to determine whether CN and PRP had a healing effect on the damage occurring in the testicular tissue after burns.

MATERIALS AND METHODS

Experimental Animals

Experimental animals to be used in the study were obtained from Atatürk University Medical and Experimental Application and Research Center (ATADEM), and the experimental process was carried out in the ATADEM. Eighty Sprague Dawley male rats were used in the study, each weighing 250-300 g and 12 weeks old. Prior to the experiment, the rats were housed at room temperature (25 ^{o}C) for 7 days, under appropriate conditions, given only feed and water in order to get adapted to the experimental environment. Eighty rats were randomly divided into 4 groups of 20. This study was carried out with the permission of Atatürk University Animal Experiments Local Ethics Committee (Date: 31.08.2021, No: 2021/194).

Establishing Burn Model in RatsIn the study, thermal damage was produced on the skin of the animals, by applying the Burn Comb Model. This was applied symmetrically without pressure on the back of the rats for 20 seconds after the special brass comb 1x2 cm in size, having 4 strings and 0.5x1 cm spaces was kept in 100 °C boiling water for 5 minutes.

Preparation of PRP

In the control group without burn, 1ml blood sample was collected into tubes containing 0.5 ml sodium citrate and centrifuged in two stages. First at 1700 rpm for 15 minutes, to seperate the red blood cell and plasma fractions, then at 3000 rpm for 5 minutes, to remove the platelet-poor plasma, and finally PRP was obtained.

Experimental Groups

Control Group: Rats were kept in a pool filled with physiological saline for 30 minutes.

Sham Group: Dermal damage was created on the skin of the rats the first day, and they were kept in 0.9% saline solution for 30 minutes.[11]

Cerium Nitrate (CN) Group: Rats were kept in bath containing 0.04 M CN for 30 minutes.

Platelet-Rich Plasma (PRP) Group: 0.1 ml of PRP was injected intradermally into the wound area of the rats with thermal damage.[12]

Semen Evaluation

One of cauda epididymidis was used to obtain semen sample for each animal. For this purpose, randomly selected cauda epididymidis was minced in Petri dish including 5 mL of physiological saline. To provide the migrations of spermatozoa from cauda epididymidis to fluid, the solution-tissue mixture was incubated in a warmed stage at 35 °C for 5 min. Following the incubation period, cauda epididymidis residue was removed by using anatomical tweezers from the Petri dish. The fluid remaining in the Petri dish was used as semen sample. Evaluation of semen was conducted using routine spermatological parameters including dead sperm rate and morphological examination of spermatozoa.

To determine the percentage of morphological abnormality of spermatozoa, the method (with a little modification by using only eosin dye instead of eosinnigrosin dye) described by Turk et al.[13] was used. Briefly, two slides for each semen sample were stained with eosin dye. Then, the slides were evaluated under light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan). Two hundred spermatozoa from each slide were examined and the numbers of spermatozoa with abnormal head were expressed as percentage.

Sperm viability was evaluated with light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan) after eosin nigrosin staining.[13] The smear was prepared for counting. A total of 200 cells were counted and the results are presented as percentages.

Taking Tissue Samples

Three weeks after burn damage, 10 rats randomly selected from each group were sacrificed under general anesthesia (Xylazine + Ketamine) and testicular tissue samples were obtained for fertility, sperm motility, histopathological and immunohistochemical examinations.

Histopathological Examinations

All testicular samples were fixed in 10% buffered formaldehyde. Tissue blocks were prepared after routine tissue follow-up steps and 4 µm thick sections were taken from the blocks. The slides were stained with Hematoxylin-Eosin (HE) and examined under a light microscope (Olympus BX51, Japan). Sections were evaluated as absent (-), mild (+), moderate (++), and severe (+++) according to histopathological findings.[14,15]

Immunohistochemical Examinations

In the immunohistochemical staining procedure, the primary antibody used was (IL-1β cat no: sc-52012, diluent ratio: 1/200 US; TNF-α cat no: sc-52746, diluent ratio: 1/200 US; iNOS: cat no: sc-7271, diluent ratio: 1/200 US) and it was incubated with the instructions for use. HRP/DAB (3,3-diaminobenzidine) was used as the chromogen, and Mayer's hematoxylin solution was used for background staining. After the staining procedure, the sections examined with a light microscope (Olympus BX51, Japan). Immunohistochemical analysis staining data were evaluated in the ImageJ analysis program.[16]

Immunofluorescence Examinations

In the immunofluorescence staining procedure, the primary antibody (8-OHdG cat no: sc-66036, diluent ratio:1/200 US; Caspase 3 cat no: 56036, diluent ratio:1/200, US) was used and incubated in accordance with the instructions for use. As secondary antibody, FITC solution (cat no: ab6717 dliuent ratio:1/500 UK) was applied to the tissues according to the instructions for use. After the staining procedure, the sections examined by fluorescence microscopy (ZEISS AXIO, Germany). Immunofluorescence analysis staining data were evaluated in the ImageJ analysis program.[17,18]

Statistical Analysis

The statistical analysis for histopathological examinations was performed using GraphPad Prisim program, with $P < .05$ considered as significant in data evaluation. Mann-Whitney U test was used for determining differences between groups. For the statistical analysis of spermatological parameters, parametric RM-one-way ANOVA was performed, followed by Tukey's tests.

RESULTS

Spermatological Examination Findings

Spermatological examinations after burns were performed, and abnormal sperm counts and membrane damage were determined and presented in Table 1.

Table 1. Evaluation of study groups in terms of spermatological parameters (x±SEM)

Group	Total abnormal sperm cell %	Membrane integrity %
Control	15.50±3.39 [a]	34.50±7.31[a]
Sham	23.50±4.92[b]	25.00±4.47[b]
CN	22.83±2.48[b]	29.50±2.34[ab]
PRP	16.66±5.16[a]	32.50±5.24[a]
p	*	*

a,b: Differences between means with different letters in the same column are significant (*: $P < .05$), CN: Cerium nitrate, PRP: Platelet-Rich-Plasma

Hematoxylin Eosin Staining Findings

Control Group: Testicular tissues displayed a normal histological structure (Figure 1A).

Sham Group: Severe degeneration and necrotic changes were noted, along with considerable thinning of the walls of seminiferous tubules, and a significant decrease in the number of Sertoli cells and spermatocytes of the testicular tissues. At the same time, significant decrease in the number of spermatozoa in the tubulus lumens and intense hyperemia in the intertubular veins and a slight lymphocyte cell infiltration were observed (Figure 1B).

CN Group: Mild thinning of the walls of the seminiferous tubules, moderate degeneration with a decrease in the number of Sertoli cells and spermatocytes were noted. At the same time, a decrease in the number of spermatozoa in the tubular lumens and moderate hyperemia in the veins in the intertubular areas were observed (Figure 1C).

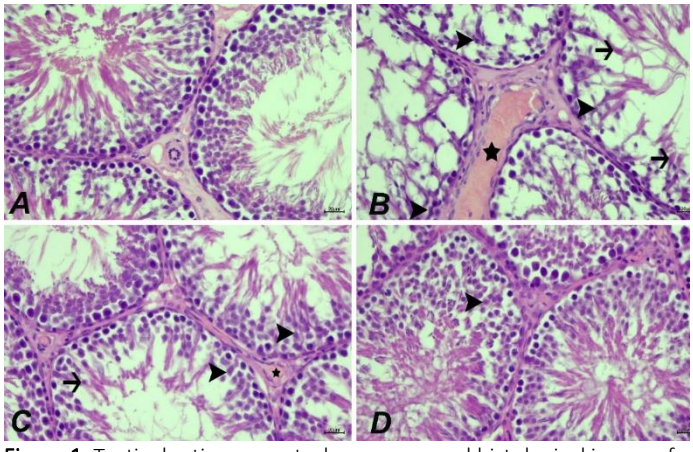

Figure 1. Testicular tissue, control group; normal histological image of testicular tissue (A), sham group; severe degeneration of spermatocytes (arrowheads), moderate necrosis of spermatocytes (arrows), severe hyperemia (star) of veins (B), CN group; moderate degeneration of spermatocytes (arrowheads), mild necrosis of spermatocytes (arrows), moderate hyperemia (star) in veins (C), PRP group; mild degeneration of spermatocytes (arrowheads) (D), H&E, Bar: 20µm

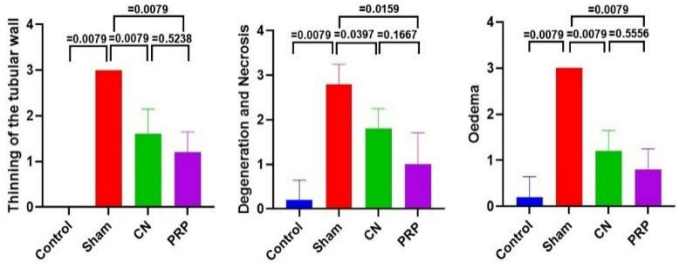

Figure 2. Scoring of histopathological findings seen in testicular tissue and statistical analysis data. Mann-Whitney U test was used for comparison between groups.

PRP Group: Mild degeneration in testicular tissue with mild decrease in Sertoli cells and spermatocyte count and moderate hyperemia in intertubular vein areas were determined (Figure 1D). Histopathological findings and scoring are presented in Figure 2.

Immunohistochemical Examination Findings

Control Group: Immunohistochemical staining of testicular tissues showed that IL-1β, TNF-α and iNOS expressions were negative (Figure 3A,4A,5A).

Sham Group: As a result of immunohistochemical staining, IL-1β, TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells. In this group, IL-1β expression was moderate, while TNF-α and iNOS expressions were significantly more severe than IL-1β expression (Figure 3B,4B,5B).

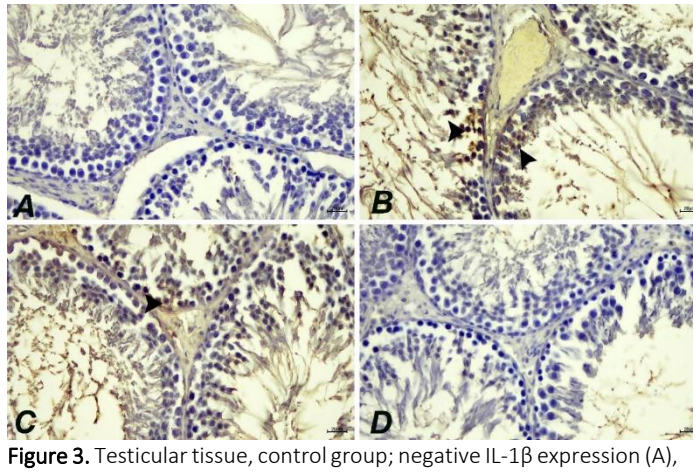

Figure 3. Testicular tissue, control group; negative IL-1β expression (A), sham group; moderate IL-1β expression (arrowheads) (B), CN group; mild IL-1β expression (arrowheads) (C), PRP group; negative IL-1β expression (D), IHC, Bar:20µm

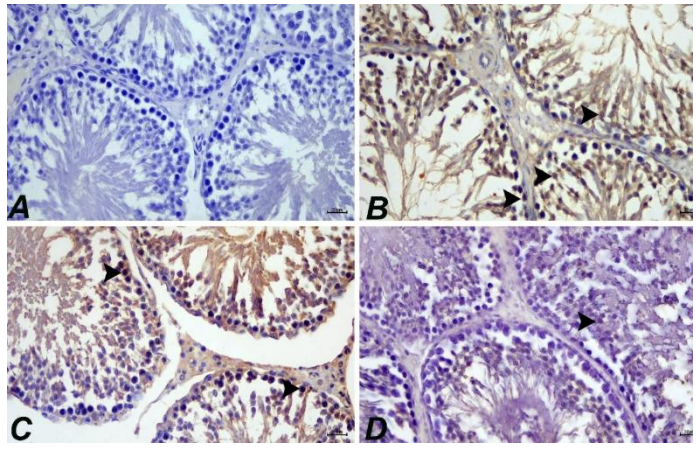

Figure 4. Testicular tissue, control group; negative TNF-α expression (A), sham group; severe TNF-α expression (arrowheads), CN group; moderate TNF-α expression (arrowheads), PRP group; slight expression of TNF-α (arrowheads), IHC, Bar:20µm

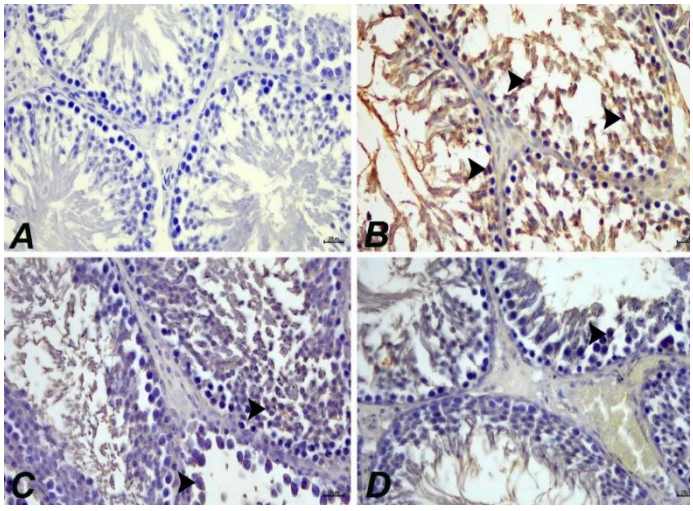

Figure 5. Testicular tissue, control group; negative iNOS expression (A), sham group; severe iNOS expression (arrowheads) (B), CN group; moderate iNOS expression (arrowheads) (C), PRP group; mild iNOS expression (arrowheads) (D), IHC, Bar: 20µm

CN Group: IL-1β, TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells. In this group, IL-1β expression was mild, while TNF-α and iNOS expressions were severe with respect to IL-1β expression (Figure 3C, 4C, 5C).

RP Group: TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells, while IL-1β expression was not observed (Figure 3D, 4D, 5D). Immunohistochemical findings scoring are presented in Figure 6.

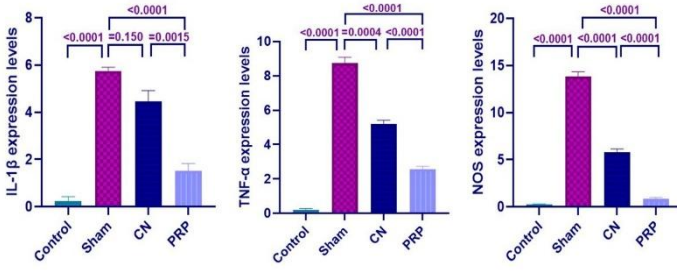

Figure 6. Immunohistochemical analysis results and statistical analysis data calculated in the ImageJ analysis program on testicular tissue. One-way ANOVA followed by Tukey's test was performed for statistical evaluation of the data in the study.

Immunofluorescence Examination Findings

Control Group: Immunofluorescence staining of testicular tissues revealed that 8-OHdG and Caspase 3 expressions were negative (Figure 7A, 8A).

Sham Goup: 8-OHdG and Caspase 3 expressions were found to be severe in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells of testicular tissues (Figure 7B, 8B).

CN Group: Moderate levels of expression of 8-OHdG and Caspase 3 were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells (Figure 7C, 8C).

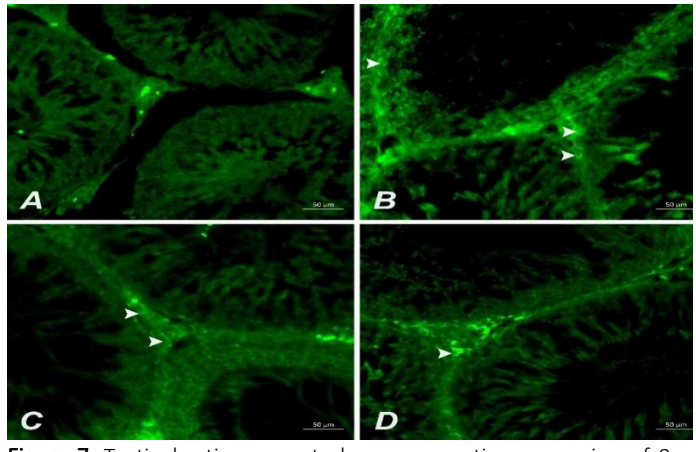

Figure 7. Testicular tissue, control group; negative expression of 8-OHdG (A), sham group; severe 8-OHdG expression (arrowheads) (B), CN group; moderate expression of 8-OHdG (arrowheads) (C), PRP group; slight expression of 8-OHdG (arrowheads) (D), IF, Bar: 50µm

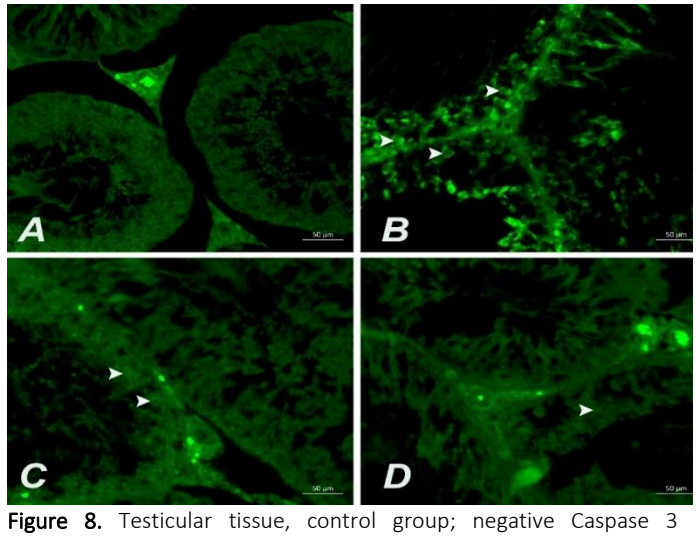

Figure 8. Testicular tissue, control group; negative Caspase 3 expression (A), sham group; severe Caspase 3 expression (arrowheads) (B), CN group; moderate Caspase 3 expression (arrowheads) (C), PRP group; light Caspase 3 expression (arrowheads) (D), IF, Bar: 50µm

PRP Group: 8-OHdG and Caspase 3 expressions were found to be mild in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells of testicular tissues (Figure 7D, 8D). Immunofluorescence findings scoring are presented in Figure 9.

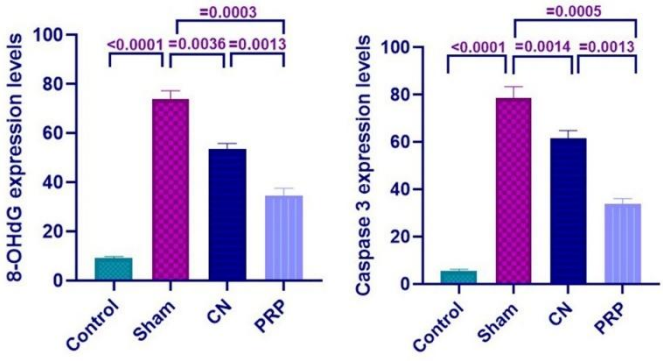

Figure 9. Immunofluorescence analysis results calculated in the ImageJ analysis program in testicular tissue and statistical analysis data. In the study, one-way ANOVA and then Tukey's test were performed for statistical evaluation of the data.

DISCUSSION

As a result of industrialization, burn is one of the the most common type of trauma today.[1,2] The term "burn" immediately reminds of dermal injury.[19,20] However, it was reported that vital organs such as liver[21] and kidney,[22] as well as testicular tissue,[23] are affected very severely due to increased body temperature in burns.

There are many studies in the literature today, using different active substances for the treatment of burn wounds.[24,25] Cerium nitrate is one of these substances, and widely used for a long time. CN was first used in burn treatment in a study conducted in 1976, demonstrating its healing properties.[26] After that, it started to be widely used in the treatment of burns.[27] PRP is another substance that was shown to be effective in the treatment of burns in recent years, though its history is not as old as CN. Many studies demonstrated its effectiveness in the treatment of burn damages.[28] In the present study where a skin burn wound was induced, it was demonstrated for the first time by histopathological, immunohistochemical, immunofluorescent and spermatological examinations that testicular tissue damage occurred due to burn and CN and PRP had a protective effect against this damage.

In studies on burns, testicular damage and impaired spermatogenesis have been observed.[23] It is seen that testicular damage findings obtained from burn studies support the spermatological findings in our study. Besides, there are studies in which many agents are used to determine the protective efficacy on testicular damage in rats.[29,30] In a study in rats, it was observed that PRP (80µl, testis local injected, single dose) application had a protective effect on spermatological parameters.[31] Similarly, the positive effects of Cerium Nitrate (CN) and Platelet-Rich Plasma (PRP) application, which we used in our study, on testicular tissue were observed.

Testicular damage and impaired spermatogenesis have been observed in studies on burns.[23] The results of these studies, indicating testicular damage, support the spermatological findings obtained in our study. There are also studies examining the protective effects of various agents on testicular damage in rats.[29,30] In a study with rats, it was observed that PRP application (80µl, intratesticular injection, single dose) had a protective effect on spermatological parameters.[31] Similarly, CN and PRP applications in our study were observed to have positive effects on testicular tissue.

A burn on the skin causes damage not only to the skin tissue, but also various tissues and organs are seriously affected, particularly the testicular tissue which is very sensitive to body temperature changes.[23] Severe damage was determined in the testicular tissues and in the seminiferous tubules of the rats induced with severe burn trauma as well as a significant reduction was observed in the number of cells responsible for sperm production and the number of mature sperm cells in the lumen.[23] In the present study too, damage to the seminiferous tubules, degeneration and necrosis of the spermatocytes were observed, indicating that the burn actually affected not only the skin tissue but also the testicular tissue at a very severe level. Although CN[32,33] and PRP[34] are widely used in the treatment of skin burns, their effects on testicular tissue have not been revealed yet in the literature. This study determined that CN and PRP treatments can be effective against histopathological lesions in testis tissue.

A severe burn trauma may cause inflammatory reactions in various tissues and organs due to the development of a systemic inflammatory response (SIRS3) in the body, although mild burn traumas generally cause local inflammatory responses.[4,5] Testicular tissue is the best example in this respect. It was reported that the inflammatory response develops very quickly after the burn due to the thermal sensitivity of the testicular tissue and its vulnerability against heat.[35] Although it is well known that a systemic inflammatory response develops in the body after a very severe burn, still the studies on testicular tissue remained very limited. A study conducted in this context reported that serum TNF alpha levels increased very severely after burns in rats.[35] It was determined in the present study, that the expression levels of IL-1β, TNF-α and iNOS increased very severely in testicular tissues due to systemic inflammatory reactions. This study revealed that the testicular tissue can also be severely affected by the systemic inflammatory reaction developing after the burn.

Today, there are many anti-inflammatory components such as CN[27,36] and PRP[37] for suppressing the systemic

inflammatory response of the body. To the best of our knowledge, there is no study in the literature investigating the anti-inflammatory effects of both CN and PRP on testicular tissues after burns, although this was demonstrated in many studies on trauma[38] and diseases.[37] This study revealed that CN and PRP have anti-inflammatory effects on testicular tissues against systemic inflammatory reactions occurring after burns.

It has been reported in many studies that free radicals released due to thermal skin damage after burns, increase oxidative stress in tissues causing DNA damage and subsequently leading cells to apoptosis depending on the severity of DNA damage.[39] In this study, where a burn wound was created/induced on the skin, increased levels of 8-OHdG expressions observed in spermatocytes indicated that testicular tissue was highly affected by oxidative stress.[40] Many studies demonstrated that both CN[41] and PRP[42,43] suppressed oxidative stress in the body. This study determined that CN and PRP active substances used in treatments, significantly reduced both 8-OHdG and Caspase 3 expression levels in spermatocytes by suppressing oxidative stress.

As a result, it was determined that the systemic inflammatory response and oxidative stress that occur in the body after burns cause inflammation in testicular tissues, as well as DNA damage and apoptosis in spermatocytes, and that CN and PRP have a protective effect against these damages. It is thought that the protective effects of CN and PRP occur by suppressing the systemic inflammatory reactions and oxidative stress that occur after burns.

Ethics Committee Approval: This study was carried out with the permission of Atatürk University Animal Experiments Local Ethics Committee (Date: 31.08.2021, Decision No: 2021/194).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – İ.B., K.A.T.K.; Design- İ.B., K.A.T.K.; Supervision- İ.B. K.A.T.K; Resources- İ.B. E.M.K., K.A.T.K.; Data Collection and/or Processing- İ.B., A.D., F.Ö., K.A.T.K.; Analysis and/or Interpretation-K.A.T.K.; Literature Search- İ.B., E.M.K.; Writing Manuscript- İ.B., E.M.K.; Critical Review- İ.B., K.A.T.K.

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