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Adipose Mesenchymal Stem Cell-Derived Exosomes Prevent Testicular Torsion Injury by Controlling Apoptosis and Necroptosis

Hilal Kabadayı Ensarioğlu[1], Fatma Bilgecan Şimşek[2], Hafize Seda Vatansever[1,3], Hasan Çayırlı[4], Aydın Şencan[4]

[1]Department of Histology and Embryology, Manisa Celal Bayar University Faculty of Medicine, Manisa, Türkiye
[2]Clinic of Pediatric Surgery, University of Health Sciences Türkiye, Gülhane Training and Research Hospital, Ankara, Türkiye
[3]DESAM Research Institute, Near East University, Nicosia, North Cyprus
[4]Department of Pediatric Surgery, Manisa Celal Bayar University Faculty of Medicine, Manisa, Türkiye

Abstract

BACKGROUND/AIMS: Testicular torsion, which occurs as a result of twisting of the spermatic cord, disrupts blood flow and causes ischemic damage, and reperfusion damage occurs when circulation is restored. Our earlier study revealed that exosomes originating from adipogenic mesenchymal stem cells (ADSCs) effectively reduce ischemia-reperfusion injury in experimental models of testicular torsion. This study investigates how these exosomes influence cell death pathways, specifically apoptosis and necroptosis.

MATERIALS AND METHODS: Twenty-one Wistar albino rats at a prepubertal stage were divided into sham, control, and treatment groups. The left testis was rotated to induce testicular torsion, then it was detorsed to allow reperfusion. The control group received ADSCs culture medium post-detorsion, while the treatment group was administered ADSC-derived exosomes (ADSC-Exos). The immunoreactivity of apoptotic (caspase 3, 8, 9, Bcl-2, Bax), necroptotic [receptor-interacting serine/threonine protein kinases 1, receptor-interacting protein 3 (RIPK1, RIP3), mixed lineage kinase domain-like (MLKL)] and spermatogonial stem cell [G-protein coupled receptor 125 (Gpr125)] markers was analyzed by indirect immunoperoxidase staining. The intensity of marker expression was evaluated by HSCORE. The results were statistically evaluated, and a p-value of <0.05 was considered significant.

RESULTS: The treatment group exhibited lower levels of caspase 3, 8, and 9 compared to the control group. Bcl-2 intensity was similar in all groups, but the Bax/Bcl-2 ratio was significantly reduced in the treatment group. The immunolabel of MLKL in the treatment group differed compared to the sham group ($p=0.053$). RIPK1 immunoreactivity was strong in the control torsion group compared to the other groups ($p=0.000$ and $p=0.254$, respectively), while RIP3 immunoreactivity was similar between the treatment group and sham group and lower compared to the control torsion group. Additionally, the highest intensity of Gpr125 was detected in the treatment group.

CONCLUSION: In the experimental testicular torsion-detorsion model, necroptosis was found more effective than apoptosis in affecting cell viability negatively. ADSC-Exos significantly reduced necroptosis in testicular cells. Additionally, exosomes had a positive effect on spermatogonial stem cells and helped prevent cell death after testicular torsion.

Keywords: Testicular torsion injury, exosomes, apoptosis, necroptosis

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ORCID IDs of the authors: H.K.E. 0000-0001-7429-1478; F.B.Ş. 0000-0003-0826-3721; H.S.V. 0000-0003-3619-5666; H.Ç. 0000-0001-8422-2417; A.Ş. 0000-0002-8892-4907.

Corresponding author: Aydın Şencan
E-mail: aydin.sencan@yahoo.com.tr
ORCID ID: orcid.org/0000-0002-8892-4907

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INTRODUCTION

Testicular torsion is a critical urological condition caused by the rotation of the testis and funiculus spermaticus that obstructs blood supply and leads to ischemia. The pathophysiological process involves ischemia caused by the twisting, followed by reperfusion once the cord is untwisted. The severity of injury is contingent on both the duration of torsion and the degree of twisting. Ischemia-reperfusion (I/R) injury initiates a cascade of pathological events, including neutrophil recruitment, the production of substance, release of proinflammatory cytokines, lipid peroxidation, cell death, anoxia, and disruptions in microvascular blood flow.[1]

Adipogenic mesenchymal stem cells (ADSCs) have shown promise in mitigating I/R injuries through their biological properties which are controlled with ADSCs.[2,3] Local application of ADSCs has been shown to preserve fertility in animal models of testicular torsion.[4,5] However, transplanted mesenchymal stromal cells often face poor survival in ischemic environments post-infarction[6], suggesting that their therapeutic effects are likely mediated by paracrine mechanisms, particularly through the release of exosomes.[7]

Exosomes are tiny vesicles (30-150 nm) surrounded by a membrane, and mediate intercellular communication by transferring molecular signals between cells. These vesicles, found in fluids like blood, urine, and semen, engage with recipient cells through various mechanisms, including ligand-receptor binding and membrane fusion.[8] Exosomes and their linked microRNAs (miRNAs) play a key role in testis development, regulating vital processes such as proliferation of spermatogonial stem cells and the meiosis of spermatocytes, while also preserving the stability of the testicular immune microenvironment.[8]

Exosomes derived from ADSC (ADSC-Exos) have shown effectiveness in reducing I/R injury in multiple organs, including the brain[9], heart[10], and kidneys.[11] These exosomes have also been shown to improve erectile function in diabetic and post-prostatectomy models.[12,13] Our previous work emphasized the protective role of ADSC-Exos against testicular torsion-induced I/R injury.[14] This investigation aims to further explore the role of ADSC-Exos, particularly how they impact cell death mechanisms, such as apoptosis and necroptosis, in a testicular torsion model.

MATERIALS AND METHODS

Cell Culture and Study Groups

The research was approved by the Experimental Animal Ethics Committee of Manisa Celal Bayar University (approval number: E-77637435-050.04.04-582692, date: 21.07.2023). Adipose-derived mesenchymal stem cells were cultured and characterized following the methods outlined in our previous study[14] Briefly, ADSCs were cultured in minimum essential medium alpha (Capricorn Scientific MEMA-XRA) containing 15% fetal bovine serum, 1% Penicillin-Streptomycin, and 1% L-Glutamine, maintained until they reached 85% confluency. The culture medium was harvested and exosomes were isolated using the miRCURY Exosome Isolation Kit (Exiqon 300102). There were three study groups, namely, sham, control, and treatment, and for each group, 7 Wistar albino male prepubertal rats were randomly assigned. In the sham group, rats underwent a left scrotal incision without any additional intervention. The sentence appears to logically misrepresent the experimental setup by suggesting treatment procedures for a control group, but since details of the study's context are necessary for further revision and such details have not been provided, the sentence remains as is. Ensure the correct experimental context before publication. After 4 hours of torsion, the testis was detorsioned, and ADSCs culture medium, without fetal bovine serum, was administered into the testicular parenchyma, followed by a 4-hour reperfusion period. In the treatment group, the same procedure was performed, but the rats were administered ADSC-Exos instead of culture medium. Following the completion of the experimental procedures, the experimental animals were euthanized and the testicular tissues were collected. These tissues were fixed in Bouin's solution, processed through a standard paraffin embedding protocol, and sectioned into 5 µm slices for further analyses.

Apoptotic and Necroptotic Evolution via Immunohistochemistry

Immunoreactivity of apoptotic markers (caspases 3, 8, 9, Bcl-2, Bax), necroptotic markers [receptor-interacting serine/threonine protein kinases 1, receptor-interacting protein 3 (RIPK1, RIP3), mixed lineage kinase domain-like (MLKL)] and spermatogonial stem cell [G-protein coupled receptor 125 (Gpr125)] markers were assessed using the indirect immunoperoxidase technique. After deparaffinization, tissue sections were treated with two changes of xylene. To rehydrate the sections, they were dipped into the graded alcohol series and rinsed with distilled water. Following this, they were dipped into phosphate buffer solution (PBS) for five minutes (min). Next, they were incubated with a trypsin solution at 37 °C for 30 min. The sections were treated with 3% hydrogen peroxide (Merck, 1.08597.2500), followed by additional washes. Blocking solution (ThermoFisher, TA-125-UB) was then incubated for 1 hour at room temperature, before being added overnight at 4 °C with primary antibodies anti-caspase 3 (Bioassay Technology Laboratory, BT-AP01199), anti-caspase 8 (Bioss antibodies, BS-0052R) anti-caspase 9 (Bioss antibodies, BS-0049R), anti-Bcl-2 (Delta Biolabs, DB001), anti-Bax (Santa Cruz Biotechnology, sc-526), anti-RIPK1 (Bioss antibodies, 5805R), anti-RIP3 (Santa Cruz Biotechnology, sc-374639), anti-MLKL (Santa Cruz Biotechnology, sc-293201) and anti-Gpr125 (abcam, ab51705) . Following PBS washing, the tissue samples were incubated with biotinylated rabbit anti-mouse secondary antibody for 30 min, followed by streptavidin-hydrogen peroxidase for an additional 30 min (ThermoFisher, TP-125-UB). After washing steps, the chromogen (DAB, 38611, ScyTek Laboratories) was applied to the slides for 5 min. Mayer's hematoxylin was added to stain the nuclei and the sections were mounted (DMM-125, Spring Bioscience). The degree of immunolabeling was evaluated by two researchers using light microscopy. The intensity of staining was graded semi-quantitatively using the H-score method. The H-score was determined using the following formula: $\sum \Pi (i + 1)$, where "I" represents the staining intensity (scored as 1, 2, or 3 for weak, moderate or strong, respectively) and "Π" denotes the percentage of cells stained at each intensity level, ranging from 0 to 100%.

Statistical Analysis

Statistical comparisons between groups were performed using the Kruskal-Wallis test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemical analyses revealed that the immunoreactivity of caspase 3 (a), caspase 8 (b) and caspase 9 (c) was reduced in the treatment (Figure 1C-a, b, c) group in contrast to the control torsion (Figure 1B-a, b, c) group, though this difference was not statistically significant

(Figure 2). Bcl-2 immunoreactivity was lower in the treatment group (Figure 1C-d), and the difference with the sham group (Figure 1A-d) was significant (p=0.044) (Figures 1, 2). MLKL intensity was similar in the treatment group (Figure 1C-h) to the control group (Figures 1B-h, 2). Higher immunoreactivity of RIPK1 was detected in the control torsion group (Figure 1B-f), compared to the sham (Figure 1A-f) and treatment groups (Figure 1C-f), (p values were 0.000 and 0.254, respectively) (Figure 2); RIP3 (g) immunoreactivity was similar in the sham (Figure 1A-g) and treatment (Figure 1C-g) groups and lower than in the control torsion group (Figure 1B-g), although this was not statistically significant (Figure 2). Bax (e) immunoreactivity was higher in the control torsion group (Figure 1B-e) compared to the sham, (Figure 1A-e) and treatment (Figure 1C-e) groups. Treatment group Gpr125 (Figure 1C-i) immunoreactivity was higher than the control (Figure 1B-i) groups and the sham (Figure 1A-i) group (p-values 0.001 and 0.017, respectively).

DISCUSSION

In recent years, stem cell therapies and exosome-based treatments, a cell-free alternative, have gained prominence as promising options for various pathological conditions due to their advantages, including superior stability, storability, absence of toxicity and aneuploidy risks, low likelihood of immune rejection, and efficient targeting of damaged areas. Stem cell therapies have also been recognized for their potential in these conditions.[15,14] The outlined effects of exosomes present a promising option for testicular torsion, offering potential benefits in preventing or mitigating I/R damage and preserving spermatogenic functions. Exosomes derived from mesenchymal stem cells modulate cellular activity and paracrine signaling by transferring proteins, lipids, mRNA, and miRNAs to recipient cells.[16]

In this study, we specifically aimed to explore the impact of ADSC-Exos particularly on apoptosis and necroptosis, in a model of testicular

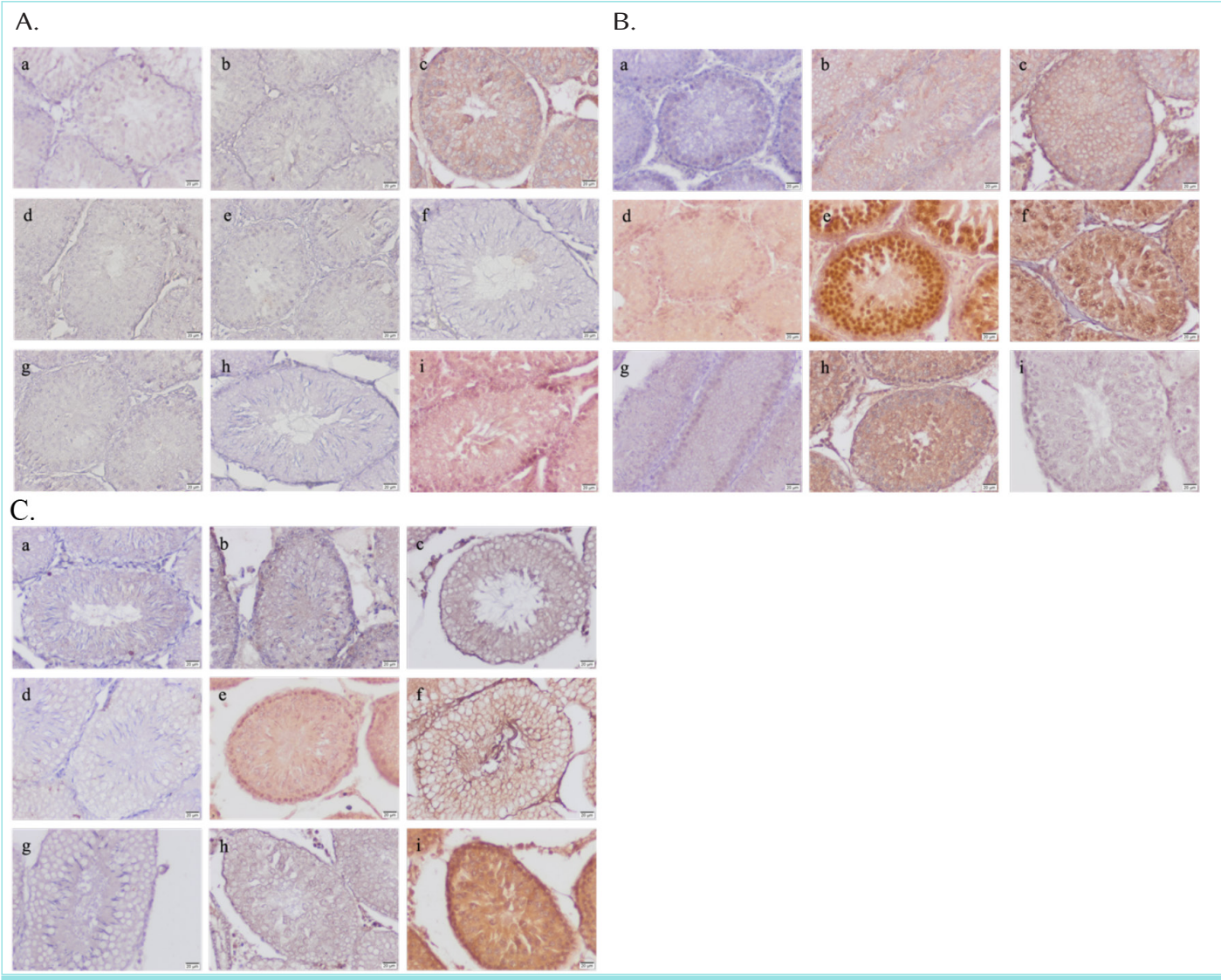

Figure 1. Distribution of caspases 3 (a), 8 (b), 9 (c), Bcl-2 (d), Bax (e), RIPK1 (f), RIP3 (g), MLKL (h), Gpr125 (i) after immunocytochemistry in the sham group (A), control torsion group (B), treatment group (C) testicles. Scale bars: 20 µm.

RIPK1: Receptor-interacting serine/threonine protein kinases 1, RIP3: Receptor-interacting protein 3, MLKL: Mixed lineage kinase domain-like, Gpr125: G-protein coupled receptor 125.

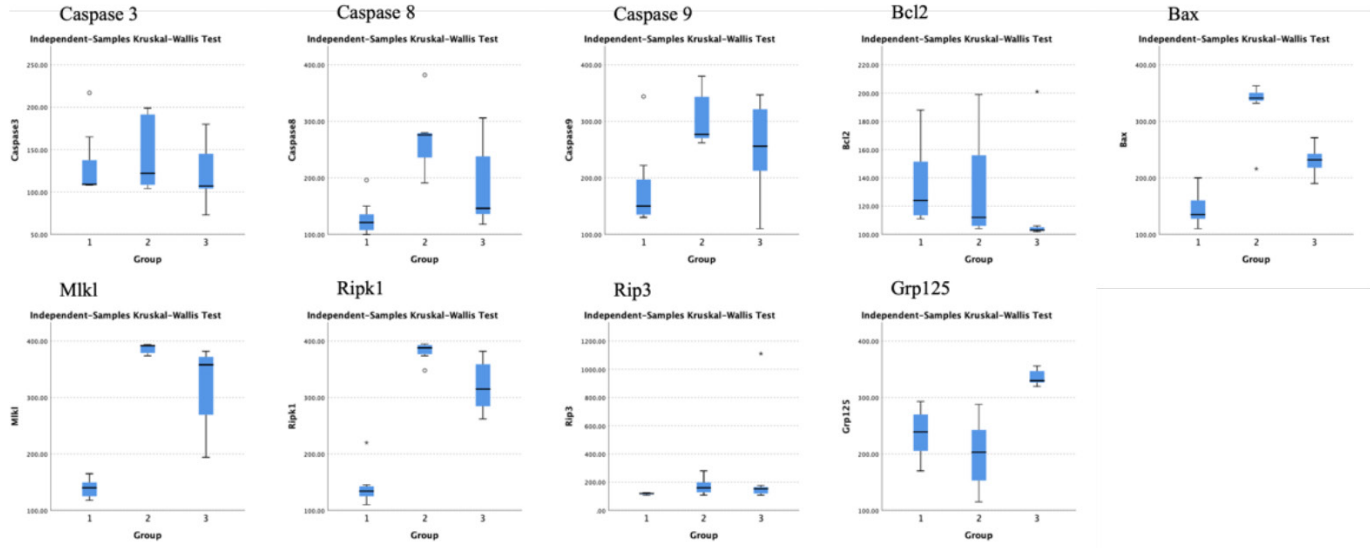

Figure 2. Statistical analyses of apoptotic (caspases 3, 8, 9, Bcl2, Bax) and necroptotic (RIPK1, RIP3 and MLKL) markers in sham (1), control (2), treatment (3) groups.

RIPK1: Receptor-interacting serine/threonine protein kinases 1, RIP3: Receptor-interacting protein 3, MLKL: Mixed lineage kinase domain-like, Gpr125: G-protein coupled receptor 125.

torsion-detorsion in rats. Previous studies have demonstrated the therapeutic potential of ADSC-Exos in various I/R injury models. For instance, Liu et al.[6] observed that ADSCs exosomes exhibited notable therapeutic potential in mitigating oxidative stress-induced ischemia/reperfusion injury, particularly through modulation of the signaling pathways, especially PI3K/AKT and MAPK/ERK1/2. Similarly, our previous study showed that ADSC-Exos effectively helped prevent ischemia reperfusion injury in a testicular torsion model, as evidenced by improvements in tissue morphology and TUNEL staining analysis.[14]

In the present study, immunocytochemistry findings showed that exosome treatment reduced the immunoreactivity of caspases 3, 8 and 9, although these changes were not statistically significant. In contrast, the difference between anti-apoptotic Bcl-2, and apoptotic Bax values was significant, suggesting a shift toward cell survival. Furthermore, the reduced intensities of necroptotic markers RIPK1, and MLKL in the treatment group suggested that exosomes can reduce the harmful effects of I/R injury by modulating key apoptotic and necroptotic pathways. These findings support the potential role of exosomes in improving tissue survival, preserving spermatogonial stem cells, and ultimately enhancing the regenerative capacity of the testicular tissue.

This study adds to the growing body of evidence that ADSC-Exos are a viable therapeutic strategy for testicular torsion-detorsion injury. Their ability to target multiple pathways involved in cell death and survival underscores their potential for broader applications in ischemic and other pathological conditions requiring precision therapy.

Study Limitations

The study relies on an animal model, which may not perfectly replicate human pathophysiology. Therefore, translating these findings directly to human clinical applications might require additional validation in larger models. Moreover, the study focuses on short-term outcomes after exosome treatment. However, the long-term effects of exosome therapy on tissue are not addressed. Additionally, investigation is needed to analyse the content of the exosomes.

CONCLUSION

Our experimental model of testicular torsion-detorsion indicated that necroptosis has a greater negative impact on cell viability than apoptosis. Furthermore, exosomes obtained from ADSCs were found to significantly mitigate necroptosis in testicular cells, while also promoting the survival of spermatogonial stem cells. Our findings indicate that exosomes have beneficial effects on cell death following testicular torsion and spermatogonial stem cell survival.

MAIN POINTS

- Exosomes derived from adipose-derived stem cells (ADSCs-Exos) offer a promising, cell-free therapeutic approach for testicular torsion by mitigating ischemia-reperfusion injury, reducing oxidative stress, and preserving spermatogenic functions.
- Exosome treatment reduced immunoreactivity of caspases 3, 8, and 9, while significantly increasing anti-apoptotic Bcl-2, and decreasing apoptotic Bax levels, indicating a shift toward cell survival. Additionally, lower expression of necroptotic markers RIPK1 and MLKL suggests a protective effect against cell death mechanisms.
- The ability of ADSCs-Exos to regulate multiple cell death and survival pathways highlights their potential for treating testicular torsion-detorsion injury.

ETHICS

Ethics Committee Approval: The research was approved by the Experimental Animal Ethics Committee of Manisa Celal Bayar University (approval number: E-77637435-050.04.04-582692, date: 21.07.2023).

Informed Consent: Not applicable.

Footnotes

Authorship Contributions

Surgical and Medical Practices: H.K.E., F.B.Ş., H.Ç., A.Ş., Concept: A.Ş, H.S.V., F.B.Ş., H.K.E., H.C., Design: A.Ş., H.S.V., H.K.E., F.B.Ş., Data Collection and/or Processing: H.K.E., F.B.Ş., H.S.V., H.Ç., A.Ş., Analysis and/or Interpretation: H.K.E., F.B.Ş., H.S.V., H.Ç., A.Ş., Literature Search: H.K.E., F.B.Ş., H.S.V., H.Ç., A.Ş., Writing: H.K.E., F.B.Ş., H.S.V., H.Ç., A.Ş.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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