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ORIGINAL RESEARCH

Protective Effect of Mesenchymal Stem Cells and Melatonin on Testicular Torsion-Induced Infertility*

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ABSTRACT

We aimed to explore the effects of systemic melatonin and intratesticular Adipose tissue-derived mesenchymal stem cells (AdMSCs) administration on rats with acute unilateral testicular torsion. Rats were randomized into Sham group (S) (n=8), torsion/detorsion group (T/D by torsion of right testis with rotated 720° counter clockwise for 3 h, then detorsion) (n=8), Melatonin group given 25 mg/kg after torsion/detorsion (M) (n=8), Adipose tissue-derived mesenchymal stem cell-treated group after torsion/detorsion (MSC) (n=8), Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion (MSC+M) (n=8). We measured MDA, Testosterone, FSH and LH levels, performed histopathological analyses in testicles, and identified SOX, VASA and Caspas-3 reactions immunohistochemically. Testosterone, FSH, LH values did not yield any significant difference between the groups. While the Johnson score in the right testis remained the lowest in T/D, the highest score was noted in the S. The T/D manifested some degenerative seminiferous tubules, abnormal spermatogenesis and maturation arrest. The degenerative appearance monitored in M, MSC and MSC+M groups persisted in some tubules, while markedly reduced degeneration was observed in some other tubules. The highest Caspase-3 expression in T/D, whereas SOX-9 expression remained significantly higher in the treatment groups. Another aspect deserving attention is that MSC were characterized by low VASA expression. Our experimental trial suggests that the torsion-induced degeneration in testicular tissue was ameliorated in all the treatment groups. Although MSC, MSC+M and M administrations decreased the torsion-induced degeneration in the testicular tissue, these treatments did not prove to be superior to each other.

Keywords: Testicular Torsion-Detorsion. Stem cell. Melatonin. Apoptosis.

Testiküler Torsiyonun İndüklediği Kısırlığa Mezenkimal Kök Hücrenin ve Melatoninin Koruyucu Etkisi

ÖZET

Sistemik melatonin ve intratestiküler adipoz doku kaynaklı mezenkimal kök hücrelerin (AdMSCs) uygulamasının akut tek taraflı testis torsiyonu olan sıçanlar üzerindeki etkilerini araştırmayı amaçladık. Sıçanlar, Sham grubu (S) (n=8), torsiyon/detorsiyon grubu (sağ testisin 3 saat boyunca saat yönünün tersine 720° döndürülerek torsiyonu ve ardından detorsiyonu ile T/D) (n=8), torsiyon/detorsiyon sonrası 25 mg/kg melatonin verilen grup (M) (n=8), torsiyon/detorsiyon sonrası adipoz doku kaynaklı mezenkimal kök hücre ile tedavi edilen grup (MSC) (n=8), torsiyon /detorsiyon sonrası melatonin ile adipoz dokusu kaynaklı mezenkimal kök hücre ile tedavi edilen grup (MSC+M) (n=8) olarak randomize edildi. MDA, Testosteron, FSH ve LH düzeyleri ölçüldü. Testislerde histopatolojik analizler yapıldı ve SOX, VASA ve Caspas-3 reaksiyonları immünohistokimyasal olarak incelendi. Testosteron, FSH, LH değerleri gruplar arasında anlamlı bir fark göstermedi. Sağ testiste Johnson skoru T/D'de en düşük seviyede kalırken, en yüksek skor S grubunda kaydedildi. T/D grubunda bazı dejeneratif seminifer tübüller, anormal spermatogenez ve olgunlaşma durması görüldü. M, MSC ve MSC+M gruplarında izlenen dejeneratif görünüm bazı tübüllerde devam ederken, diğer bazı tübüllerde belirgin şekilde azalmış olduğu gözlendi. En yüksek Kaspaz-3 ekspresyonu T/D'de görülürken, SOX-9 ekspresyonu tedavi gruplarında anlamlı derecede yüksek kalmıştır. Dikkat edilmesi gereken bir diğer husus da MSC'nin düşük VASA ekspresyonu ile karakterize olmasıdır. Deneysel çalışmamız, testis dokusunda torsiyona bağlı dejenerasyonun tüm tedavi gruplarında iyileştiğini göstermektedir. MSC, MSC+M ve M uygulamaları testis dokusunda torsiyona bağlı dejenerasyonu azaltmış olsa da, bu tedavilerin birbirlerine üstünlüğü kanıtlanmamıştır.

Anahtar Kelimeler: Testiküler Torsiyon-Detorsiyon. Kök hücre. Melatonin. Apoptoz.

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Testicular torsion is defined as the reduction or complete absence of blood flow to the testis and related structures owing to the twisting of the spermatic cord around its own axis[1]. Testicular torsion decreases the total amount of antioxidants while increasing the volume of oxidants and the oxidative stress index[2]. Free oxygen radicals induced by ischemia-reperfusion (I/R) injury contribute to testicular damage[3]. Since spermatozoa harbor high levels of unsaturated fatty acids in their plasma membranes, they are highly susceptible to oxidative stress and to lipid peroxidation in particular[4].

Stem cells refer to undifferentiated cells which potentially generate similar cells and transform into various somatic cells[5]. Adipose tissue-derived mesenchymal stem cells (AdMSCs) can reportedly prevent testicular torsion-induced germ cell damage and trigger spermatogenesis in the seminiferous tubules[6]. There is mounting clinical evidence that the AdMSCs transplanted locally may inhibit I/R-induced intrinsic apoptosis, alleviate the oxidative stress of damaged testicular tissue, and transdifferentiate into spermatogenetic cells[7].

Melatonin is an endogenous antioxidant synthesized from tissues such as the pineal gland, retina, gastrointestinal tract, and testis[8]. It has the potential to repair testicular injury and stimulate spermatogenesis in T/D rats by means of the inhibition of oxidative stress, restoration of sexual hormone homeostasis, expression of growth factors[9]. Many studies have shown that the protective effects of melatonin on testicular T/D rat models[3,10-12]. Melatonin is capable of reducing the release of inflammatory cytokines, enhancing the proliferation capacity of mesenchymal stem cells, blocking the *in vitro* and *in vivo* apoptosis of MSCs. In addition, it promotes the protective and anti-apoptotic mechanisms of MSCs through regulating the expression of ROS-generating factors and antioxidant genes[13].

One of the main causes of infertility is ischaemic damage resulting from testicular torsion. To date, the effects of melatonin and stem cells on testicular torsion have been studied in separate contexts. Several lines of evidence suggest that melatonin promotes the proliferation of mesenchymal stem cells and inhibits their apoptosis. Against this background, this study aims to investigate and compare the effects of melatonin and stem cell therapy on rats with acute unilateral testicular torsion.

Material and Method

Experimental design

Ethical approval was granted by the Pamukkale University Ethical Commission for Animal Experiments (PAUHADYEK-2019/03). Male Wistar-Albino rats which were 8 weeks old and weighed 350-400 g were randomized into five groups. For general anaesthesia, xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (90 mg/kg) were given intraperitoneally. In the Sham group (S), only surgical incision was performed (n=8). In the torsion/detorsion group (T/D), after the right testis was delivered from the scrotum, it was rotated 720º in a clockwise direction and fixed with bulldog clamp and maintained in this position. At the end of 3 h, it was detorsioned and replaced into the scrotum, and the gubernaculum was closed up by a silk suture (n= 8). The Melatonin Group (M), as soon as detorsion was performed 3 h after the testicular torsion, a single-dose (25mg/kg) melatonin was administered intraperitoneally (n=8)[14]. The MSC Group (MSC), AdMSCs were injected locally multiple sites to the right testis at a dose of $3x10^5$ cells, following the T/D procedure (n=8)[6]. The MSC+M Group (MSC+M), the 25 mg/kg melatonin and $3x10^5$ AdMSCs after the T/D procedure (n=8) (Figure 1).

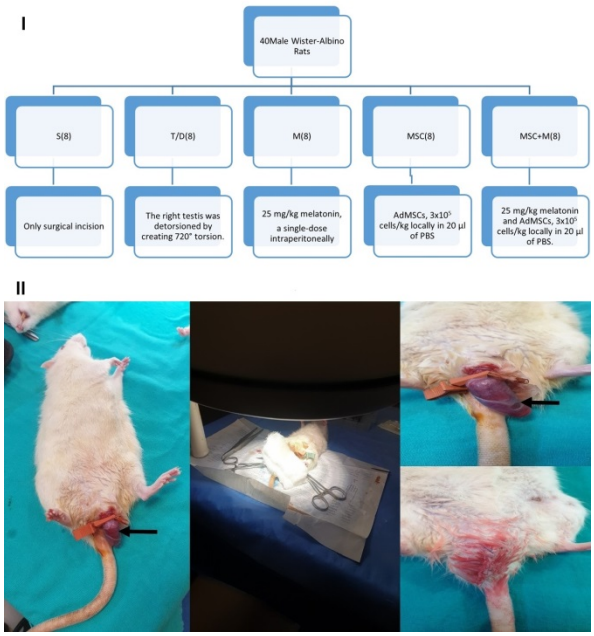

Figure 1. I. *Flowchart of testicular torsion-detorsion and study design. S; Sham group, T/D; torsion/detorsion group, M; Melatonin group given 25 mg/kg after torsion/detorsion , MSC ; Adipose tissue-derived mesenchymal stem cell-treated group after torsion/detorsion, MSC+M; Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion.* ***II.*** *The torsion/detorsion group, following the opening of the scrotum, the right testis was rotated 720º in a clockwise direction and maintained in this position. At the end of 3 h, it was detorsioned and placed into the scrotum. (Arrow: Torsion of right testis)*

AdMSCs Transplantation

We used the AdMSCs obtained and frozen in the project numbered 2016BSP001. In this project, MSCs were isolated from the perirenal adipose tissue of male rat. Adipose tissue was transferred to the laboratory in phosphate-buffered saline (PBS) and quickly minced into small pieces. After washing with PBS, the tissue was incubated with 0.1% collagenase enzyme. The high-glucose Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin were then left in the incubator (5% CO2, 37°C). After thawing, cells were monitored for proliferation. Cells that reached 70% confluence were removed with 0.25% trypsin and cell counts were performed with trypan blue. For the MSC and MSC+M groups, viable $3x10^5$ AdMSCs were then injected locally into the right testis.

ELISA Method

Seven days after the AdMSCs injection, blood was taken by a cardiac puncture. The serum specimens of the groups were analyzed using a commercial kit (BT Laboratory-Shanghai, China). FSH (mIU/mL), LH (mIU/mL), Testosterone (ng/mL) and MDA (nmol/mL) levels were calculated using serum absorbance values.

Histopathology

The testicles dissected were punctured with an injector, immersed in Bouin's fixative. The tissues were hydrated under running water. They were fixed in 10% formaldehyde, and tissue follow-up was performed. Tissues were embedded in paraffin. Transverse sections (5µm) were processed for hematoxylin-eosin (H&E) staining.

Seminiferous tubular area, epithelial thickness and Modified Johnsen Score System (MJS)

H&E stained sections were visualized at 20X to calculate epithelial thickness(µm) and tubular areas(μm^2). Ten randomly-selected fields and 4 seminiferous tubules per field were measured for groups[15]. MJS quantifies spermatogenesis along a scale ranging from 1 to 10. In this scoring system, 9 or 10 is assigned for normal histology, 8 for hypospermatogenesis, 3-7 for maturation arrest, 2 for germinal cell aplasia (Sertoli cells only), and 1 for tubular fibrosis. 30 tubule germinal epithelium was evaluated for each testis, and MJS was calculated for each testis[16].

1. No seminiferous epithelium.
2. No germinal cells. Sertoli cells only.
3. Spermatogonia only.
4. No spermatids or spermatozoa, few spermatocytes.
5. No spermatids or spermatozoa, many spermatocytes.
6. No spermatozoa and late spermatids, few early spermatids.
7. No spermatozoa and late spermatids, many early spermatids.
8. Fewer than 5 spermatozoa per tubule, few late spermatids.
9. Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium.
10. Full spermatogenesis.

Immunohistochemical Staining

The sections were deparaffinized and rehydrated. The endogenous peroxidase activity was eliminated after treatment with H2O2: Methanol mixture. The sections hydrated were blocked with serum blocking solution added. The primary antibodies were added for overnight incubation (SOX-9 (Bioss antibodies lot: AA12301405, dilution: 1:200, USA), VASA (Bioassaytechnology, lot:20190628dilution: 1:200, China), Caspase-3 (Elabscience, lot:EC5206, dilution:1:200, USA). The sections were treated with secondary antibodies (Thermo scientific lot: PHLS772). The chromogen dye was treated with 3,3'Diaminobenzidine (DAB). Hematoxylin was performed for counterstaining. The expressions were evaluated by assigning a semi-quantitative H-score [H SCORE=$\sum$Pi (I+1)], where I represents staining intensity (0=no expression, 1= mild, 2= moderate, 3= strong), and Pi expresses the percentage of cells stained for each intensity.

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) staining

Using BioTnA Biotech kit, the sections passed through alcohol series. They were hydrated and immersed in 3% of H2O2. Triton X-100 was incubated with Tween-PBS Solution. Proteinase-K was added and allowed to stand at room temperature, it was equilibrated in pre-incubation buffer solution. TdT Reaction Mixture was incubated at 37°C in a humid environment. They were incubated in anti-mouse DIG solution at 37°C in a humidified atmosphere and was kept in HRP-labeled polymer solution. The sections were exposed to DAB. Hematoxylin was used for counterstaining. 200 spermatogenic cells were counted in five randomly-fields. Brown-stained cells were identified as TUNEL-positive. Apoptotic index (AI) was calculated as the percentage of TUNEL-positive cells divided by the total number of cells.

Statistical Analysis

The collected data were analyzed with SPSS v.25 (IBM SPSS Statistics 25 software) package program. Continuous variables were presented as mean ± standard deviation, median (minimum-maximum values) and categorical variables as numbers and percentages. Shapiro Wilk test was used for testing normality of the data. Kruskal-Wallis Variance Analysis were carried out for independent groups. The significance level was established at $p<0.05$.

Results

Characterization of MSCs

The high titer of CD29, CD90, CD54 (99.36%, 93.68%, 99.07%, respectively) surface markers and the low titer of CD45, CD71 (0.64% and 1.99%, respectively) counterparts reveal that we obtained MSCs (Figure 2I). As far as our differentiation experiments are concerned, stem cells are found to be capable of differentiating into adipogenic, chondrogenic and osteogenic cells (Figure 2II).

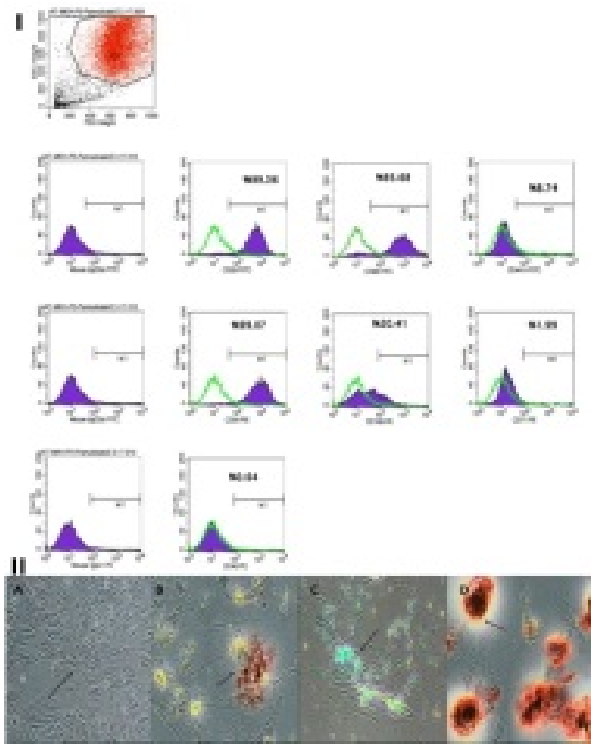

Figure 2.
I. *Representative flow cytometry histograms of Adipose tissue-derived mesenchymal stem cells (AdMSCs). from rat. The cells showed high titer expression of CD29, CD90, CD54 (99.36%, 93.68%, 99.07%, respectively) surface markers, and low titer of expression of CD45, CD71 (0.64% and 1.99%, respectively) (FACS analysis).* ***II.*** *Characterization of rat AdMSCs. A, Attached large spindle shaped, fibroblast-like morphology in passage 3, (arrow: AdMSCs). B, AdMSCs rat cultivated in adipogenic medium that were stained with Oil Red O at day 21 after induction (arrow: adipogenic differentiation), C, AdMSCs of rat cultivated in chondrogenic medium and stained with archean blue (arrow: chondrogenic differentiation) D, AdMSCs of rat cultivated in osteogenic medium and stained with alizarin red (arrow: osteogenic differentiation) ×200, inverted microscope.*

Blood FSH, LH, Testosterone and MDA Levels

Between-group analysis did not yield a significant difference in relation to MDA levels. Although MDA levels turned out to be low in MSC and MSC+M groups, they were not statistically significant (p>0.05). Significant difference was not noted between the Testosterone, FSH, LH values of the groups (Table I).

Table I. Blood levels of MDA, TESTOSTERONE, FSH, LH in different groups.

		S	T/D	M	MSC	MSC+M	*p*
MDA (nmol/ml)	**Mean±SD**	1.9±0.38	2.03±0.15	2.15±0.18	1.63±0.59	1.63±0.45	0.103
	Median (Min-Max)	2.03(1.16-2.22)	2.03(1.87-2.18)	2.15(1.81-2.39)	1.61(0.8-2.44)	1.67(1.06-2.14)	
TESTOSTERONE (ng/ml)	**Mean±SD**	42.79±0.14	42.75±0.3	42.8±0.3	42.96±0.3	43.15±0.11	0.12
	Median (Min-Max)	42.83(42.57-42.99)	42.77(42.24-43.19)	42.86(42.31-43.2)	43.08(42.51-43.24)	43.16(43.03-43.24)	
FSH (mIU/ml)	**Mean±SD**	13.49±2.44	16.62±2.76	17.33±1.49	16.26±3.46	16.83±0.54	0.116
	Median (Min-Max)	12.99(10.92-16.94)	16.56(12.27-21.66)	17.85(15.34-18.99)	16.58(11.12-21.49)	16.88(16.12-17.43)	
LH (mIU/ml)	**Mean±SD**	85.98±17.49	94.7±10.18	81.05±18.92	91.68±12.16	87.88±7.72	0.674
	Median (Min-Max)	94.29(53.12-97.93)	93.42(83.24-112.6)	76.78(53.56-103.6)	88.58(75.67-110.2)	86.43(80.42-98.22)	

S; Sham group, T/D; torsion/detorsion group, M; Melatonin group given 25 mg/kg after torsion/detorsion, MSC; Adipose tissue derived mesenchymal stem cell-treated group after torsion/detorsion, MSC+M; Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion, p<0.05.

Tubule Area, Epithelial Thickness, MJS Findings

Considering the comparison of the right testicular tubule areas, the tubule area of the S group was significantly higher than that of the T/D group. The tubule areas were significantly higher in the S group than in the T/D group, the MSC group and the MSC+M group. Some increase was detected in the right testicular tubule area in the M group relative to the T/D group, though not statistically significant. The right and left testis MJS in groups were observed to be significantly higher in the S group (9.93±0.1) than the other groups. Another notable finding is that the right testis in the T/D group yielded the lowest MJS score (4.9±1.07). MJS scores proved to be the highest in the S group (9.93±0.1), followed by the M group (7.83±1.4), the MSC+M group (6.25±1.43), and the MSC group (5.97±1.11). (Figure 3).

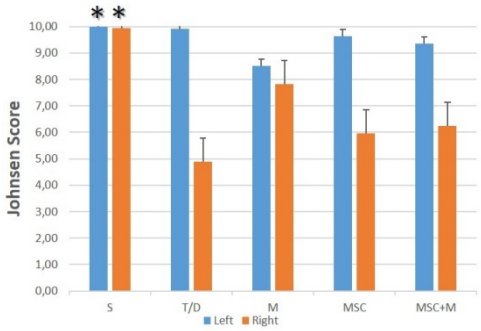

Figure 3.

*Johnsen's score was determined in each group at each time period after surgery. Values are shown as mean±SD. Sign (*) is indicated as significance with the Sham and the other groups. (*p<0.05)*

H&E Staining Findings

The seminiferous tube epithelium revealed normal morphology and thickness in the S group. Normal spermatogenesis was apparent throughout the epithelium. In the T/D group, the diameter of the seminiferous tubule was found to be reduced, and abnormal spermatogenesis and maturation arrest were evident. T/D group manifested intercellular cystic changes, intercellular ruptures, vacuolization, and degenerative changes in the seminiferous tubule cells. The damaged tubules were characterized by low cell density, and we noted the thinning of epithelium, a large lumen formation, congestion in testicular vessels. In the M group was similar to the S group rather than the T/D group, yet multinucleated giant cells were detected in some tubules. In the MSC and MSC+M groups, even though poor cellular connections were established in some tubules, we also observed intact ones. The giant cells detected in the M group did not appear in the MSC and MSC+M groups (Figure 4A).

Immunohistochemical Findings

Caspase-3 expression was markedly strong in the T/D group. It was significantly enhanced in the T/D group in comparison to the other groups ($p<0.05$). While Caspase-3 expression was significantly higher in MSC and MSC+M groups than the S group ($p<0.05$). The presence of Caspase-3 expression was significantly reduced in the M group relative to the MSC and MSC+M groups ($p<0.05$). SOX-9 expression was significantly lower in the S group than the treatment groups ($p<0.05$). A significant difference did not yield between the treatment groups. Whereas the S, T/D and M groups, did not differ significantly between themselves, they featured a significantly higher level of VASA expression than the MSC and MSC+M groups ($p<0.05$). The M group was characterized by the highest VASA expression. The M group yielded a significantly higher level of VASA expression than MSC and MSC+M groups ($p<0.05$) (Figure 4).

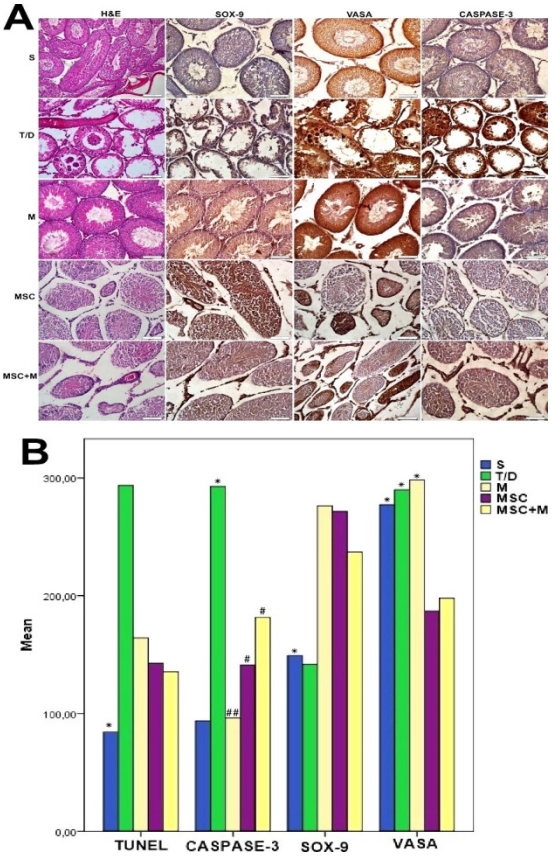

Figure 4.

A. *Hematoxylin and eosin staining and immunohistochemical localization of SOX-9, VASA, CASPASE-3 in all groups. S; Sham group, T/D; torsion/detorsion group, M; Melatonin group given 25 mg/kg after torsion/detorsion, MSC; Adipose tissue-derived mesenchymal stem cell-treated group after torsion/detorsion, MSC+M; Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion. H&E, hematoxylin and eosin, ×200.* ***B.*** *Apoptotic index of TUNEL and HSCORE values of CASPASE-3, SOX-9, VASA immunoreactivity in groups. Data are expressed as means ± SD, Statistical significance was determined by one-way ANOVA. S; Sham group, T/D; torsion/detorsion group, M; Melatonin group given 25 mg/kg after torsion/detorsion , MSC ; Adipose tissue-derived mesenchymal stem cell-treated group after torsion/detorsion, MSC+M; Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion. TUNEL:*p <0.05 compared with the other groups. CASPASE-3: *p <0.05 compared with the other groups. #p <0.05 compared with the S group. ## p <0.05 compared with MSC and MSC+M. SOX-9: *p <0.05 compared with the treatment groups. VASA: *p <0.05 compared with the MSC and MSC+M group*

TUNEL Staining Findings

In the T/D group, seminiferous tubules, Leydig cells, blood vessels and connective tissue yielded a strong positive reaction. In M, MSC and MSC+M groups, had negative expression in seminiferous tubules, while Leydig cells, blood vessels and connective tissue in the interstitial area showed moderate positive reaction. In relation to the between-group comparisons, AI of the S group was significantly lower than the others ($p<0.05$). Although the M, MSC, MSC+M groups yielded significantly higher AI than the S group, this value turned out to be significantly lower than the T/D group ($p<0.05$). However, the between-group comparisons of the treatment groups revealed no significant difference ($p>0.05$) (Figure 4-5).

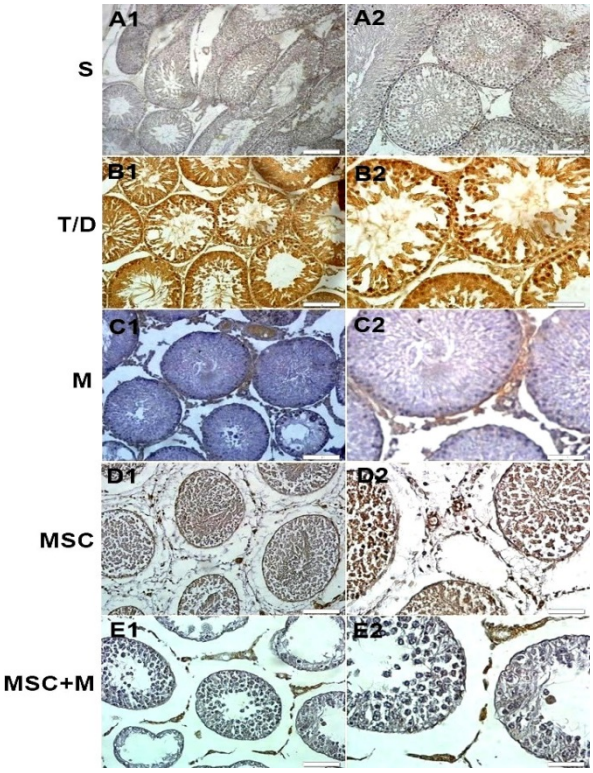

Figure 5.
Apoptotic cell labeling with TUNEL in the groups. Apoptotic cell was increased in T/D group S; Sham group, T/D; torsion/detorsion group, M; Melatonin group given 25 mg/kg after torsion/detorsion MSC; Adipose tissue-derived mesenchymal stem cell-treated group after torsion/detorsion, MSC+M; Adipose tissue-derived mesenchymal stem cell-treated group with melatonin after torsion/detorsion. (A, B1, C1, D1, E1-X200) (A2, B2, C2, D2, E2-X400)

Discussion and Conclusion

The treatments of M, MSC and MSC+M did not affect the hormonal functions of rats with T/D, at the end of 7 days. We observed that MDA decreased in treatment groups although it was not statistically significant. Caspase-3 expression and the number of apoptotic cells caused by T/D significantly decreased in the treatment groups.

The MSCs derived from diverse tissues were administered to rats with testicular T/D, significantly inhibiting MDA concentration in the treatment groups[9,17,18]. Consistent with these studies, the MDA levels were suppressed on the 7th day in the MSC group in our study, yet the level of this decrease was not statistically significant. Melatonin not only protects cell membrane but also the nucleus and organelles against free radical-mediated oxidative damage[19]. Infertile patients reportedly manifest lower serum and seminal plasma levels of melatonin[20]. A study in which the rats with testicular T/D were subjected to zinc- and melatonin-supplemented administration reported that this treatment suppressed oxidative stress and decreased inhibin-B levels. Melatonin administered before testicular T/D procedure restored blood and tissue Glutathione and MDA levels[21]. An experimental trial which investigated the melatonin on testis in young and elderly rats indicated that the melatonin reduced MDA levels and elevated glutathione and testosterone levels in elderly rats. This trial also highlighted that melatonin treatment inhibited the formation of apoptotic cells and promoting sperm motility[22]. In another study, the histopathological investigations revealed no significant improvement in MDA levels[14]. Our study, the melatonin elevated the MDA levels to some extent, though not significantly, on the 7th day in rats with testicular T/D, rather than inhibiting them. The lower MDA levels in the MSC+M group compared to the T/D and M groups demonstrate that MSCs proved effective in suppressing oxidative damage, independently of melatonin.

Testicular T/D is responsible for reduced diameter of seminiferous tubules[21]. Melatonin exerts a protective effect in testicular torsion[12], Ischemia-Reperfusion[23] and cyclophosphamide-induced toxicity[24]. It has been documented that the presence of melatonin promotes the establishment of spermatogenesis in testicular T/D damage[14]. Another study revealed that the administration of melatonin increased tubule diameter. Considering the mean tubule diameter and MJS in the melatonin-injected experimental group, the resulting values were close to those of the control group. In apoptotic cells, similar results to the control group were obtained in the melatonin-administered group in comparison to the group with damaged testicular tissue.[19] An experimental trial reported that 25 µg/kg melatonin brought about some increase in the diameter of the tubules in rats, but this increase was not statistically significant[14]. In our study, the tubule area and epithelial thickness were the highest in the S group and the lowest in the T/D group. The treatment groups increased their tubule areas and epithelial

diameters, but no significant difference was established. Recently, there is accumulating clinical evidence that stem cell therapy leads to significantly higher MJS[9,17,18]. Our findings revealed that, despite the unaffected hormone levels, melatonin contributed to the structural regulation of testis. As far as MJS results are concerned, pronounced improvement was observable in the right testis in the treatment groups compared to the T/D group, and the most apparent improvement was noted in the M group.

In an empirical study, the melatonin and colchicine repaired the apoptotic effects, the formation of giant cells was detected in the melatonin- and colchicine-administered groups[3]. Note that the presence of multinuclear giant cells was established only in the M group in our study, but one surprising finding to emerge from our analysis was that these giant cells were not available in the MSC+M group. AdMSCs are capable of integrating into interstitial and seminiferous tubule areas, including the testicular blood wall, where they synthesize various growth factors, inhibit oxidative stress, and help to maintain normal hormone levels[18]. The histological improvements occurring in the seminiferous tubules and interstitial areas in the experimental groups of our study broadly support the work of other relevant studies.

AdMSCs improved ischemia-related organ dysfunction, as well as they have anti-inflammatory and immunomodulatory capacity for ischemia-reperfusion induced organ dysfunction[25]. MSCs are involved in immunosuppressive activities through paracrine interactions[26]. In a comprehensive study, injected stromal vascular fraction (SVF) cells significantly integrated into the interstitial region and up-regulated the expression of fibroblast growth factor and stem cell factor in the seminiferous tubules. SVF cells also helped to minimize the damage in germ cells, yet Sox-9 expression did not differ significantly between the groups[9]. According to our dataset, although Sox-9 expression did not yield a significant difference between the S and T/D groups, significantly lower expression was observed in these two groups than the treatment groups. In the aforementioned study, although VASA and P450(+) cell expression were significantly higher in the treatment groups than their T/D counterpart, these expressions still remained lower than the S group[9]. In contrast, VASA expression in our study remained remarkably high in the S, T/D, and M groups, while this volume was low in the MSC-injected groups.

Melatonin rescued spermatogenesis by down-regulating the expression of apoptosis-related genes and raising the total antioxidant capacity in the testicular tissue[27]. Melatonin suppressed the radioiodine-induced apoptotic effects and its activity of impairing sperm quality[28]. The results of TUNEL, Caspase-3 and Bax of a study documented MSC-mediated intrinsic apoptosis[17]. Another study showed that neutrophil infiltration, ROS and germ cell apoptosis were reduced in the MSC group. Germ cell apoptosis was induced by testicular torsion, notably on the 1st and 3rd days. However, the proportion of apoptotic cells in the MSC group on the 1st and 3rd days are close to the control group. The TUNEL-positive cells in the torsion group were similar to the MSC and control groups after the 7th day[26]. Our study, the apoptotic cells in the spermatogenic series cells of the treatment groups showed a decreasing tendency in the tissues dissected on the 7th day. AI in the treatment groups remained significantly lower than the T/D group, though not as low as the S group. However, the efficacy of the treatment did not imply a significant superiority between the groups. Our Caspase-3 and TUNEL findings suggest that the underlying mechanisms of these treatments may prove effective in regulating the intrinsic apoptotic pathway. Chen *et al*.[25] investigated the effect of AdMSCs administered through the tail vein and melatonin given in repeated doses in ischemia reperfusion injury. According to this study, M and AdMSC is the most effective treatment[25]. In our study, the treatment groups improved T/D damage. However, we did not find a significant difference between the groups.

Our study had some limitations. We could not label the presence of MSC in the testes to show that they were correctly placed. The long-term effect of AdMSC on prevention of infertility could not be examined, because we finished the study in seven days.

Our experimental trial suggests that the torsion-induced degeneration in testicular tissue was ameliorated in all the treatment groups. But the co-administration of melatonin and MSCs did not prove to be superior to the other treatment groups with respect to VASA, SOX- 9, Caspase-3 expression, and apoptotic effects.

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Idea and design: N.Ç., G.N.; Data collection and processing: N.Ç., G.N.; Analysis and interpretation of data: N.Ç., G.N., G.A.M.; Writing of significant parts of the article: N.Ç., G.N., G.A.M.

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The authors declare no confict of interests.

References

1. Gul SS, Gurgul S, Uysal M, Erdemir F. The Protective Effects of Pulsed Magnetic Field and Melatonin on Testis Torsion and Detorsion Induced Rats Indicated by Scintigraphy, Positron Emission Tomography/Computed Tomography and Histopathological Methods. Urol J. 2018;15(6):387-96.
2. Abasiyanik A, Dağdönderen L. Beneficial effects of melatonin compared with allopurinol in experimental testicular torsion. J Pediatr Surg. 2004;39(8):1238-1241. h
3. Sekmenli T, Gunduz M, Öztürk B, Karabağlı P, Ciftci I, Tekin G, et al. The effects of melatonin and colchicine on ischemia-reperfusion injury in experimental rat testicular torsion model. J Pediatr Surg. 2017;52(4):582-6.
4. Morielli T, O'Flaherty C. Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. Reproduction 2015;149(1):113-23.
5. Karimaghai N, Tamadon A, Rahmanifar F, Mehrabani D, Raayat Jahromi A, Zare S, et al. Spermatogenesis after transplantation of adipose tissue-derived mesenchymal stem cells in busulfan-induced azoospermic hamster. Iran J Basic Med Sci. 2018;21(7):660-7.
6. Hsiao CH, Ji AT, Chang CC, Cheng CJ, Lee LM, Ho JH. Local injection of mesenchymal stem cells protects testicular torsion-induced germ cell injury. Stem Cell Res Ther. 2015;6(1):113.
7. Cakici C, Buyrukcu B, Duruksu G, Haliloglu AH, Aksoy A, Isık A, et al. Recovery of fertility in azoospermia rats after injection of adipose-tissue-derived mesenchymal stem cells: the sperm generation. Biomed Res Int. 2013;529589.
8. Reiter RJ, Tan DX, Rosales-Corral S, Manchester LC. The universal nature, unequal distribution and antioxidant functions of melatonin and its derivatives. Mini Rev Med Chem. 2013;13(3):373-84.
9. Zhou L, Song K, Xu L, Zhao F, Tian H, Zhou C, et al. Protective Effects of Uncultured Adipose-Derived Stromal Vascular Fraction on Testicular Injury Induced by Torsion-Detorsion in Rats. Stem Cells Transl Med. 2019;8(4):383-91.
10. Yurtçu M, Abasiyanik A, Avunduk MC, Muhtaroğlu S. Effects of melatonin on spermatogenesis and testicular ischemia-reperfusion injury after unilateral testicular torsion-detorsion. Journal of pediatric surgery 2008;43:1873–1878.
11. Jeong SJ, Choi WS, Chung JS, Baek M, Hong SK, Choi H. Preventive effects of cyclosporine combined with prednisolone and melatonin on contralateral testicular damage after ipsilateral torsion-detorsion in pubertal and adult rats. The Journal of urology 2010;184:790–796. https://doi.org/10.1016/j.juro.2010.03.109
12. Ekici S, Doğan Ekici AI, Öztürk G, Benli Aksungar F, Sinanoğlu O, Turan G, Lüleci N. Comparison of melatonin and ozone in the prevention of reperfusion injury following unilateral testicular torsion in rats. Urology 2012;80(4):899-6.
13. Hu C, Li L. Melatonin plays critical role in mesenchymal stem cell-based regenerative medicine in vitro and in vivo. Stem Cell Res Ther. 2019;10(1):13.
14. Mirhoseini M, Talebpour Amiri F, Karimpour Malekshah AA, Rezanejad Gatabi Z, Ghaffari E. Protective effects of melatonin on testis histology following acute torsion-detorsion in rats. Int J Reprod Biomed. 2017;15(3):141-6.
15. Mouro VGS, Menezes TP, Lima GDA, Domingues RR, Souza ACF, Oliveira JA, et al. How Bad Is Aluminum Exposure to Reproductive Parameters in Rats? Biol Trace Elem Res. 2018;183(2):314-324.
16. Johnsen SG. Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones 1970;1(1):2-25.
17. Hsiao CH, Ji AT, Chang CC, Chien MH, Lee LM, Ho JH. Mesenchymal stem cells restore the sperm motility from testicular torsion-detorsion injury by regulation of glucose metabolism in sperm. Stem Cell Res Ther. 2019;10(1):270.
18. Siregar S, Sasongko Noegroho B, Adriansjah R, Mustafa A, Wijayanti Z. Intratesticular Human Adipose-Derived Stem Cell (hADSC) Transplantation Decreased Oxidative Stress in Testicular Torsion Model of Wistar Rat. Res Rep Urol. 2021;13:1-8.
19. Karabulut D, Akın A, Sayan M, Kaymak E, Öztürk E, and Yakan B. Effects of Melatonin Against Thioacetamide-Induced Testicular Toxicity in Rats. International Journal of Morphology 2020;38:1455-1462.
20. Yu K, Deng SL, Sun TC, Li YY, Liu YX. Melatonin Regulates the Synthesis of Steroid Hormones on Male Reproduction: A Review. Molecules. 2018;23(2):447.
21. Semercioz A, Baltaci AK, Mogulkoc R, Avunduk MC. Effect of Zinc and Melatonin on Oxidative Stress and Serum Inhibin-B Levels in a Rat Testicular Torsion-Detorsion Model. Biochem Genet. 2017;55(5-6):395-409.
22. Muratoğlu S, Akarca Dizakar OS, Keskin Aktan A, Ömeroğlu S, Akbulut KG. The protective role of melatonin and curcumin in the testis of young and aged rats. Andrologia. 2019;51(3).
23. Parlaktas BS, Atilgan D, Ozyurt H, Gencten Y, Akbas A, Erdemir F, Uluocak N. The biochemical effects of ischemia-reperfusion injury in the ipsilateral and contralateral testes of rats and the protective role of melatonin. Asian J Androl. 2014;16(2):314-8.
24. Chabra A, Shokrzadeh M, Naghshvar F, Salehi F, Ahmadi A. Melatonin ameliorates oxidative stress and reproductive toxicity induced by cyclophosphamide in male mice. Hum Exp Toxicol. 2014;33(2):185-5.
25. Chen YT, Chuang FC, Yang CC, Chiang JY, Sung PH, Chu YC, Huang CR, Huang KH, Yip HK. Combined melatonin-adipose derived mesenchymal stem cells therapy effectively protected the testis from testicular torsion-induced ischemia-reperfusion injury. Stem cell research & therapy 2021;12:370.
26. Zhong L, Yang M, Zou X, Du T, Xu H, Sun J. Human umbilical cord multipotent mesenchymal stromal cells alleviate acute ischemia-reperfusion injury of spermatogenic cells via reducing inflammatory response and oxidative stress. Stem Cell Res Ther. 2020;11(1):294.
27. Liu ZM, Zhu WP, Huang DP, Bai PM.Effects of melatonin on oxidative stress and apoptosis-related gene signaling pathways following testicular torsion in rats. Zhonghua Nan Ke Xue. 2019;25(5):309-4.
28. Cebi Sen C, Yumusak N, Atilgan HI, Sadic M, Koca G, Korkmaz M. The protective effect of melatonin on sperm quality in rat after radioiodine treatment. Andrologia. 2018. https://doi.org/10.1111/and.12962