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Therapeutic Effects of Mesenchymal Stem Cell Conditioned Medium in Rat Varicocele Model

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Purpose: This study aimed to examine the therapeutic effects of injection of conditioned medium of adipose-derived mesenchymal stem cells (ADMSC-CM) in a surgically created varicocele model in comparison with varicocelectomy.
Materials and Methods: Twenty-eight male Wistar Albino rats were randomly divided into four groups: sham group, varicocele group, varicocelectomy group, and ADMSC-CM injection group. Sperm parameters were analyzed in samples taken from the epididymis after treatment. Malondialdehyde and superoxide dismutase (SOD) levels in blood samples were examined by biochemical analysis. The testicular tissues were stained with hematoxylin-eosin for histological examination (Johnsen's Score). Additionally, Western Blot analyzes were performed to detect Claudin-11 levels, the functional protein of the blood-testis barrier, in testicular tissues.
Results: Varicocelectomy and ADMSC-CM treatments significantly improved mean sperm parameters (concentration, progressive motility, motility, normal sperm morphology) ($p\leq0.05$ for all). Both treatment groups had increased SOD levels along with a decrease in malondialdehyde levels ($p\leq0.05$ for all). No significant difference was observed between the ADMSC-CM group and the varicocelectomy group in preserving normal testicular histology according to Johnsen's Score ($p=0.114$). Levels of Claudin-11 were significantly higher in the varicocelectomy and ADMSC-CM groups compared to the varicocele group ($p\leq0.05$ for all).
Conclusions: The therapeutic effects of ADMSC-CM in varicocele model may involve secretion of anti-inflammatory and regenerative factors from ADMSC. ADMSC-CM injection appears to be a promising new strategy in the treatment of varicocele.

Keywords: Claudins; Culture media, conditioned; Mesenchymal stem cells; Infertility; Varicocele

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INTRODUCTION

Varicocele is the most common medically treatable cause of male infertility, with an incidence up to 15% in healthy men [1,2]. Varicoceles are present in 35% to 44% of males experiencing primary infertility and 45% to 81% of men experiencing secondary infertility [3]. Men with varicocele exhibit reduced sperm con-

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centration and motility compared to those without the condition [4], even if the varicocele is low grade [2]. For men with clinical varicoceles and aberrant semen parameters, the American Urological Association (AUA) and the American Society for Reproductive Medicine (ASRM) [5], the European Association of Urology (EAU) [6], and the European Academy of Andrology (EAA) [7] all advise varicocelectomy. However, significant number of infertile men with a clinical varicocele and aberrant semen parameters who undergo varicocelectomy cannot achieve spontaneous pregnancy, which is the ultimate goal of the surgical treatment. It is also uncertain if varicocelectomy is necessary in situations of isolated oligozoospermia, isolated asthenozoospermia, or isolated teratozoospermia [8]. Despite the acknowledged role of varicocele in the pathophysiology of male infertility, there is little data in the literature to draw firm conclusions on the impact of varicocelectomy on semen quality.

In the recent years, the efficacy of mesenchymal stem cell (MSC) therapy has been extensively studied for numerous medical conditions, in both basic science researches and clinical trials [9-11]. Although it was initially suggested that MSCs migrate to the damaged tissues, become localized in these areas, begin to differentiate, and eventually replace damaged cells; recent studies have shown that the number of MSCs transplanted into damaged tissue or organ is quite low. Therefore, the therapeutic benefits of MSCs are attributed to their release of biologically active factors [12].

Considering these paracrine effects of MSCs, a new therapeutic approach associated with MSC administration has been introduced. This method is the use of ambient containing biologically active factors and extracellular vesicles, generally called mesenchymal stem cell conditioned medium (MSC-CM) [13]. As a result of proteomic analyzes with MSC-CMs, more than 100 proteins (including cytokines, chemokines, and growth factors) of the conditioned medium have been identified with anti-inflammatory, anti-apoptotic, anti-fibrotic and regenerative effects [14,15]. MSC-CMs have been applied in different disease models and the results showed that its functions are similar to those of MSCs, including neuroprotection, immunosuppression, tissue repair and anti-inflammatory effects [16,17].

The objective of this study was to assess the therapeutic efficacy of MSC-CM in a rat model of varicocele, comparing its effects with those of varicocelectomy. Our hypothesis posited that MSC-CM administration could serve as a viable alternative treatment to varicocelectomy for varicocele. Specifically, we aimed to investigate the therapeutic efficacy of MSC-CM through comprehensive evaluations of testicular tissue histopathology, the expression of the blood-testis barrier (BTB) protein Claudin-11, sperm parameters, and the levels of oxidant/antioxidant markers.

MATERIALS AND METHODS

1. Animal experimental groups and surgery

This study has been conducted in accordance with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. All surgical interventions were performed by single surgeon with intraperitoneal ketamine 0.9 g/kg and xylazin 10 mg/kg combined general anesthesia. A total of 28 male Wistar Albino rats (9–10 weeks old) were divided into the following groups:

1) Sham group (n=7): the rats had midline laparotomy incision and its closure.
2) Varicocele group (n=7): a varicocele model was created by partial ligation of the left renal vein [18]. Briefly, a midline laparotomy incision was made to expose the upper left abdominal quadrant. The left kidney, left renal vein, left adrenal vein, and left spermatic vein were exposed by removing the adhering fat and connective tissue by blunt dissection. The renal vein was partially occluded with a 4-0 silk suture. The suture was fastened around the vein along with a 0.85 mm-diameter metal wire. The metal wire was removed after the suture was knotted to create a consistent partial blockage with an exterior renal vein diameter that is constant at about 1 mm (Fig. 1).
3) Varicocele+varicocelectomy group (n=7): the varicocele of the rats was reversed by the ligation of the internal spermatic vein, 3 months after the varicocele model had been created.
4) Varicocele+conditioned medium of adipose-derived mesenchymal stem cells (ADMSC-CM) (n=7): The rats with varicocele received ADMSC-CM injections into their left testicles, 3 months after the varicocele model has been created. A total of 50 μL of ADMSC-CM was injected with 30G needles, twice a week for the following 4 weeks.

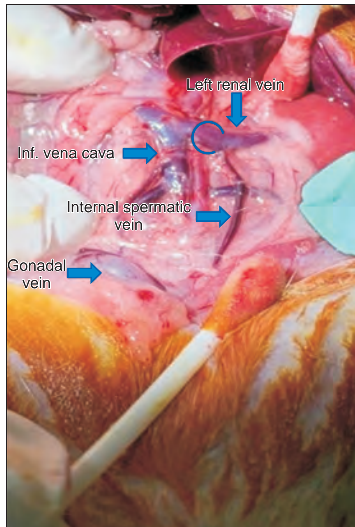


Fig. 1. The exposure of the left renal vein, gonadal vein, and left spermatic vein during creating varicocele model. The circle represents the location of the 4-0 silk suture which was fastened around the left renal vein along with a 0.85 mm-diameter metal wire. The metal wire was removed after the suture was knotted to create a consistent partial blockage with an exterior renal vein diameter that is constant at about 1 mm.

2. Ethics statement

Animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Istanbul Medipol University (IACUC approval No. E38828770-77202-2083).

3. Isolation and culture of MSCs

ADMSCs were purchased from Kocaeli University Stem Cell and Gene Therapies Research and Application Center in frozen vials containing 1×10^6 cells. Cell isolation and characterization analyzes were performed by the same center. MSCs were obtained from adipose tissue located in the inguinal region of male Wistar Albino rats. The adipose tissue was washed with Hanks balanced salt solution, containing 5% penicillin-streptomycin and was cut into 1 mm^3 pieces on a cold plate. The separated pieces were digested in 1 mg/mL collagenase I (BS163, Biosharp) solution for 30 minutes at 37 °C to obtain the cell suspension. The suspension was then centrifuged at 2,000 rpm for 5 minutes. The cell pellet was seeded into 100 mm wells. To culture cells, low-glucose DMEM (11885084, Gibco) supplemented with 10% fetal bovine serum (F7524, Sigma Aldrich), 2% L-glutamine (G6392, Sigma Aldrich), and 1% antibiotic-antimycotic solution (A5955, Sigma Aldrich) were used. MSCs were cultured at 37 °C and 5% CO_2 for 5 days. When cells reached 70% to 80% confluence, they were collected and passaged using 0.05% trypsin (25300054, Gibco). At least 3 passages were obtained before cellular characterization. Passage 3 MSCs were used in all experiments [19].

4. Flow cytometry analysis

Flow cytometry analysis was performed for the characterization of MSCs, taking into account the guidelines set by the International Society for Cellular Therapy (ISCT). ISCT guidelines stated that MSCs can express surface markers CD44, CD29, CD54, and CD90 but lack expression of CD45, CD31, and CD3 [20]. Cells that reached the third passage were trypsinized and washed twice with phosphate-buffered saline. For analysis of each marker, 1×10^5 cells were counted and separated. In the analyses, CD29-FITC (11-0299-42, Invitrogen), CD90-FITC (105305, Biolegend), CD44-FITC (OX-29, Invitrogen), CD45-PE (HIS24, Abcam), CD54-PE (116107, Biolegend), CD31-PE (25031082, Thermo Fischer), CD3-FITC (MA16623, Invitrogen) conjugated antibodies were used. Cells were incubated with antibodies for 20 minutes at 4 °C in the dark, were suspended in 500 μL FACS buffer after washing, and analyzed by Flow Cytometer (Accuri C6, BD Biosciences).

5. Preparation of conditioned medium of ADMSCs

Following characterization analyses, isolated MSCs were incubated at 37 °C and 5% CO_2. Cell medium was replaced daily with medium containing less fetal bovine serum (7%-5%-2%-0%) and cultured until reaching 80% prevalence. Therefore, by inhibiting the emergence of toxins, undesirable proteins, and oxidative stress-related alterations, cells' ability to adapt to a serum-free environment was ensured [21]. When 80% confluency was reached, the medium above the cells was collected and passed through 0.22 μm filters. The filtered medium was transferred to insulin syringes.

6. Malondialdehyde and superoxide dismutase analysis

At the end of the treatment procedures, the animals were sacrificed and blood samples were collected. Superoxide dismutase (SOD) (MBS036924, MyBiosource) and malondialdehyde (MDA) (MBS268427, MyBiosource) levels were measured using the manufacturer's protocols.

7. Sperm isolation and assessment of sperm function parameters

The epididymis was dissected and placed in Tyrode's buffer pre-warmed to 37 °C. After 10 minutes of incubation, sperm parameters were evaluated by a Macler Counting Chamber (Sefi Medical Instruments LTD). For analysis, 100 frames of each sample were counted under a light microscope with a 20× magnification. The results are given as million/mL for concentration and as percent for total+progressive motility. Diff-Quick (Abris+) staining was performed for sperm morphology analysis. Stained slides were analyzed by light microscopy (Nikon Eclipse, Nikon® Instruments Inc.) at 100× magnification. Overall, 100 sperm cells were analyzed for calculation of morphologically normal sperm and abnormal sperm (head, neck, and tail defects) percentages [22].

8. Histopathological evaluation

Left testicles were removed and divided into two pieces. One half was fixed in Bouin's solution for 24 hours and the other half was put on dry ice for Western Blot analysis. After being dehydrated through ascending alcohol series (70%, 90%, 96%, 100%), the tissues were cleared with xylene. Tissues were kept in paraffin in a 60 °C oven for 1 night. Afterwards, the tissues were embedded in paraffin and 5 μm thick sections were taken with microtome (HM 340E, Thermo Scientific). For Johnsen's Score, the slides were stained with hematoxylin-eosin (H&E) (BPK 4088-2, Empire Genomics) and imaged with Nikon Eclipse (Nikon® Instruments Inc.). Each slide was assessed in 10 similar fields containing 50 seminiferous tubules with 20× magnification [23]. Each tubule was scored between 1 and 10, and analyzed as follows: 10, full spermatogenesis; 9, many late spermatids; disorganized tubular epithelium; 8, few late spermatids; 7, no late spermatids, many early spermatids; 6, no late spermatids, few early spermatids, arrest of spermatogenesis at the spermatid stage, disturbance of spermatid differentiation; 5, no spermatids, many spermatocytes; 4, no spermatids, few spermatocytes, arrest of spermatogenesis at the primary spermatocyte stage; 3, spermatogonia only; 2, no germ cells, Sertoli cells only; 1, no seminiferous epithelial cells, tubular sclerosis.

9. Western blot analysis

Tissue samples taken from the other half of the testicular tissue were homogenized with the RIPA lysis buffer system (sc-24948, Santa Cruz Biotechnology). The homogenate was centrifuged at 10,000 g for 15 minutes at 4 °C and the supernatant was collected. Protein quantification was calculated using the Qubit 4.0 Fluorometer device (Invitrogen Life Technologies Corp.). Samples with 1.25% 2-mercaptoethanol, 4X laemmli buffer solution (161-0747, Biorad LifeSciences Research) and ddH_2O were denatured at 90 °C for 5 minutes. Mini-Protean TGX Precast protein gel (4569033, Biorad LifeSciences Research) was placed in a vertical gel electrophoresis tank (1658004, Biorad LifeSciences Research). After adding the buffer solution (1610732, Biorad Life Sciences Research) required for electrophoresis, samples calculated to contain 30 μg protein were loaded into each well and the gel was run at 200 volts for approximately 40 minutes. Proteins were transferred from the gel to the polyvinylidene fluoride membrane using a Trans-blot turbo transfer system (1704155, Biorad Life Sciences Research). To block nonspecific binding, membranes were incubated with 5% skim milk powder (sc-2325, Santa Cruz Biotechnology) prepared with Tris-buffered saline (TBS-T) for 1 hour at room temperature. Blocked membranes were incubated with anti-Claudin-11 (1:1,000 μL, 36–4,500 μL; Thermo Fisher Scientific) at 4 °C for 1 night. After washing the membranes with TBS-T 3 times for 5 minutes, they were incubated with secondary antibody for 1 hour at room temperature. After the membranes were washed 3 times for 5 minutes with TBS-T, they were incubated with ECL Western imaging solution (1705060, BioRad Life Sciences Research) for 5 minutes. Imaging was performed with the Chemidoc MP imaging system (1708280, BioRad Life Sciences Research). Protein amount control was achieved using β-actin (sc-47778, Santa Cruz Biotechnology). The captured images were analyzed with the Image J program.

10. Statistical analyzes

Data are expressed as mean±standard error mean. All data obtained in this study were analyzed with GraphPad Prism version 9.0 (GraphPad Software). Student's t-test was used to assess differences in measured variables between the two groups. The Mann–Whitney U test was used to evaluate the differences in sperm parameters between the groups. One way ANOVA multiple comparisons test was used for multiple comparisons. The results were considered statistically sig-

nificant at p<0.05.

RESULTS

1. Characterization of conditioned medium of adipose-derived mesenchymal stem cells

For cell size and granularity analyses, the R1 area was gated by performing forward scatter and side scatter analyses. Negative expressions of CD3, CD31, and CD45 along with positive expressions of CD29, CD54, CD90, and CD44 were confirmed in accordance with the ISCT identification criteria ADMSCs (Fig. 2) [20].

2. Serum malondialdehyde and superoxide dismutase levels

MDA levels, which are a marker of oxidative stress, were determined as 0.36±0.03 in the sham group, 2.61±0.16 in the varicocele group, 1.43±0.08 in the varicocele+varicocelectomy group, and 1.07±0.03 in the varicocele+ADMSC-CM group. Antioxidation-related SOD levels were determined as 9.86±0.29 in the sham group, 2.18±0.21 in the varicocele group, 6.74±0.33 in the varicocele+varicocelectomy group, and 7.41±0.54 in the varicocele+ADMSC-CM group (Table 1).

The lowest MDA level was detected in the sham group and the highest in the varicocele group. No significant difference was observed between the varicocele+varicocelectomy group and varicocele+ADMSC-CM groups (p=0.055). MDA levels were found to be significantly lower in the varicocele+varicocelectomy and varicocele+ADMSC-CM groups compared with the varicocele group (p<0.0001 for all). The highest SOD level was detected in the sham group and the lowest in the varicocele group. No significant difference was observed between the varicocele+varicocelectomy group and varicocele+ADMSC-CM groups (p=0.57). SOD levels were detected to be significantly higher in the groups treated

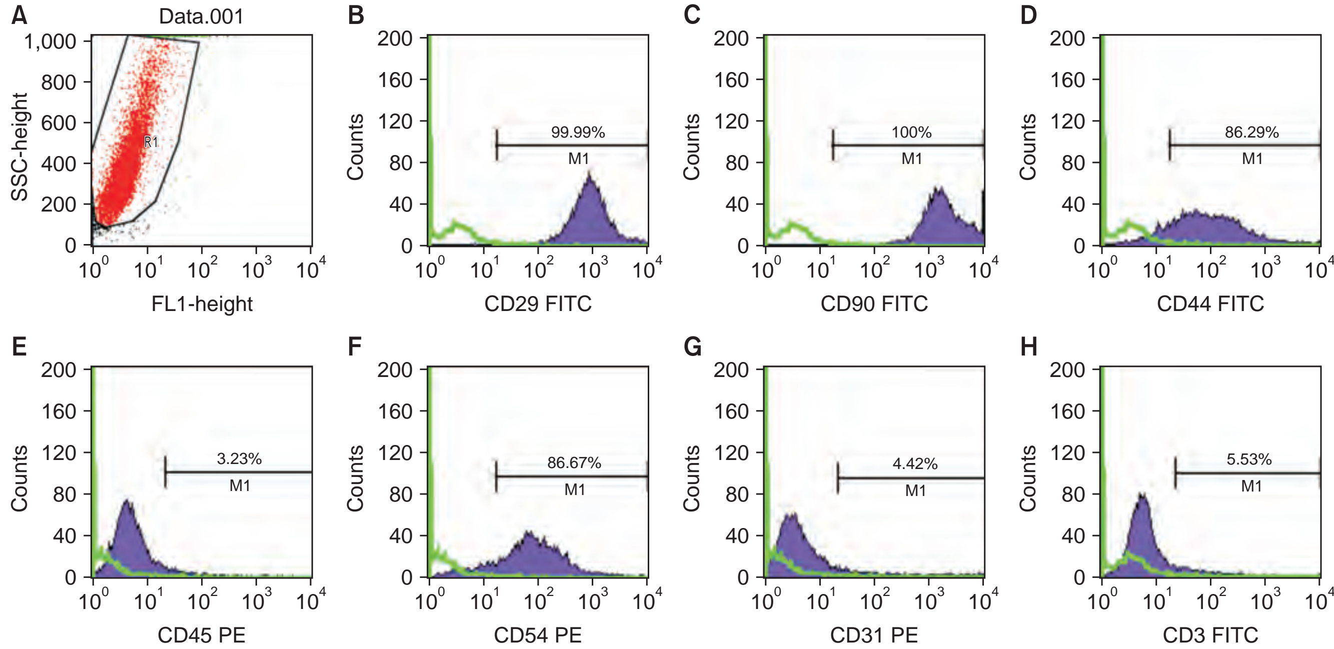


Fig. 2. (A) Red area is live cells, (B) CD29 expression level 99.99%, (C) CD90 expression level 100%, (D) CD44 expression level 86.29%, (E) CD45 expression level 3.23%, (F) CD54 expression level 86.67%, (G) CD31 expression level 4.42%, and (H) CD3 expression level 5.53%. SSC: side scatter, M1: M1 macrophages, FITC: fluorescein isothiocyanate, PE: phycoerythrin.

Table 1. Serum malondialdehyde (MDA) and superoxide dismutase (SOD) levels of the groups

Variable	Sham	Varicocele	Varicocele+Varicocelectomy	Varicocele+ADMSC-CM
MDA (μmol/L)	0.36±0.03	2.61±0.16	1.43±0.08	1.07±0.03
SOD (μmol/L)	9.86±0.29	2.18±0.21	6.74±0.33	7.41±0.54

Values are presented as mean±standard error mean.
ADMSC-CM: conditioned medium of adipose-derived mesen-chymal stem cell.

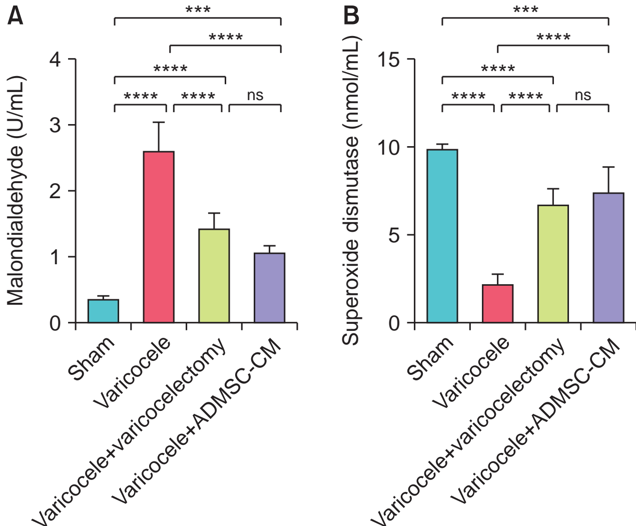


Fig. 3. (A) Statistical comparison of malondialdehyde levels of groups, (B) Statistical comparison of superoxide dismutase levels of groups. ADMSC-CM: conditioned medium of adipose-derived mesenchymal stem cell. ns: p>0.05, ***p≤0.001, ****p≤0.0001.

with varicocelectomy and ADMSC-CM compared with the varicocele group (p<0.0001 for all) (Fig. 3).

3. Sperm parameters

Sperm concentration values of the groups were determined as 10^6/mL, and values of progressive motility, motility, normal and abnormal morphology were determined as percentages (Table 2). The lowest sperm concentration was detected in the varicocele group. Sperm concentrations of the groups treated with varicocelectomy (p=0.0058) and ADMSC-CM (p<0.0001) were significantly higher than the varicocele group. However, no significant difference was observed between the two treatment groups (p=0.0525). There was no significant difference in sperm concentrations between the treatment groups and the sham group (Fig. 4A). The progressive motility rate was significantly higher in the ADMS-CM-treated group compared with

Table 2. Sperm concentration, progressive motility, motility, and normal morphology rates (%)

Variable	Sham	Varicocele	Varicocele+varicocelectomy	Varicocele+ADMSC-CM
Sperm concentration (10^6/mL)	47±6.36	7.75±2.32	34±5.25	53.42±5.23
Progressive motility (%)	25.56±7.58	0.85±0.85	12.6±2.52	23.61±5.38
Motility (%)	48.77±7.93	26.14±8.29	44.84±9.45	48.93±3.18
Normal morphology (%)	88.71±1.19	16.29±4.32	59.14±11.31	82.14±4

Values are presented as mean±standard error mean.
ADMSC-CM: conditioned medium of adipose-derived mesenchymal stem cell.

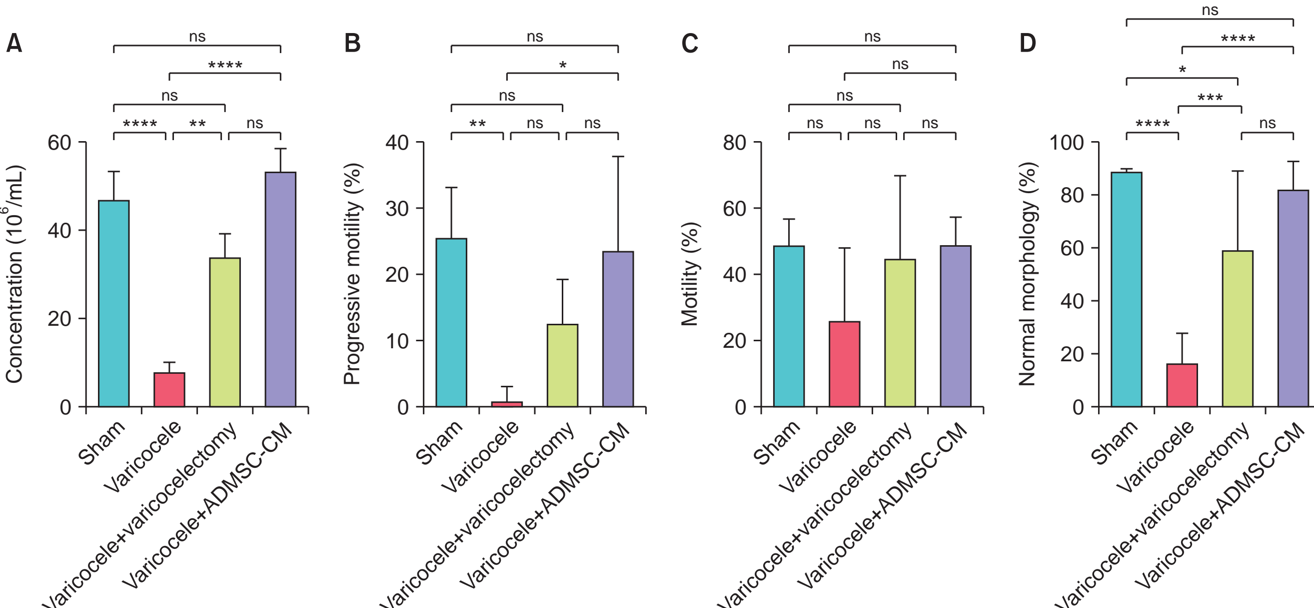


Fig. 4. Comparison of sperm parameters between sham, varicocele, varicocele+varicocelectomy, and varicocele+ADMSC-CM groups. (A) Sperm concentration, (B) progressive motility, (C) motility, and (D) normal morphology. ADMSC-CM: conditioned medium of adipose-derived mesenchymal stem cell. ns: p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

the varicocele group (p=0.0141). No significant difference was observed between the varicocelectomy-treated group and the varicocele group (Fig. 4B). There was no significant difference between the groups in terms of total motility (Fig. 4C). In the control group, sperm cells showed normal morphology (Fig. 5A). The groups with the highest proportion of sperm with normal morphology were the sham group and the ADMSC-CM-treated group. The rate of sperm with normal morphology was found to be significantly higher in both the varicocelectomy (p=0.0004) and ADMSC-CM (p<0.0001) treated groups, compared to the varicocele group (Fig. 4D). The lowest rate of sperm with normal morphology was detected in the varicocele group (Fig. 5B–5D). No significant difference was observed in terms of abnormal morphology between the groups treated with varicocelectomy and ADMSC-CM.

4. Histopathological analysis of testes

Johnsen's Score was determined as 8.99±0.38 in the sham group, 3.42±0.20 in the varicocele group, 5.08±0.88 in the varicocelectomy-treated group and 6.82±0.35 in the ADMSC-CM-treated group (Table 3). No significant difference was observed in the levels of preservation in testicular histology between the varicocelectomy group and the ADMSC-CM-treated group (p=0.114). In the sham group, seminiferous epithelium showed normal morphology (Fig. 6A). In the varicocele group, most of seminiferous tubules showed germ cell disorganization and hyaline degenerations. Also, some seminiferous tubules were free of germ cells and were containing only Sertoli cells (Fig. 6B). Varicocelectomy preserved the number of seminiferous epithelial layers, but openings between germ cells were observed (Fig. 6C). The seminiferous tubule morphology in the treatment groups exhibited similar morphology to those in the sham group (Fig. 6D).

5. Claudin-11expression levels

The lowest amount of Claudin-11 was detected in the varicocele group. Claudin-11 levels in the groups treated with varicocelectomy (p=0.0196) and ADMSC-CM (p=0.0368) were recorded to be significantly higher, compared to the varicocele group. However, no signifi-

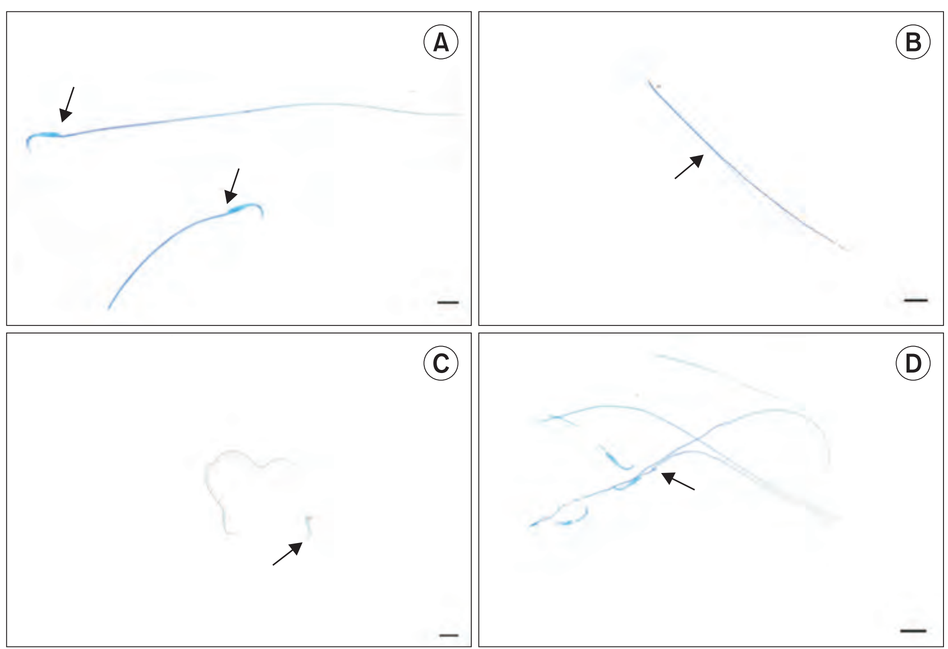


Fig. 5. Microphotographs showing morphologically normal sperm and sperm defects. (A) normal morphology, (B) headless tail, (C) detached head, and (D) cytoplasmic droplet (indicated with black arrows). Maginification: ×1,000; oil objective. Bar=10 µm.

Table 3. Johnsen's Score for each group

Variable	Sham	Varicocele	Varicocele+varicocelectomy	Varicocele+ADMSC-CM
Johnsen's score	8.99±0.38	3.42±0.20	5.08±0.88	6.82±0.35

Values are presented as mean±standard error mean.
ADMSC-CM: conditioned medium of adipose-derived mesenchymal stem cell.

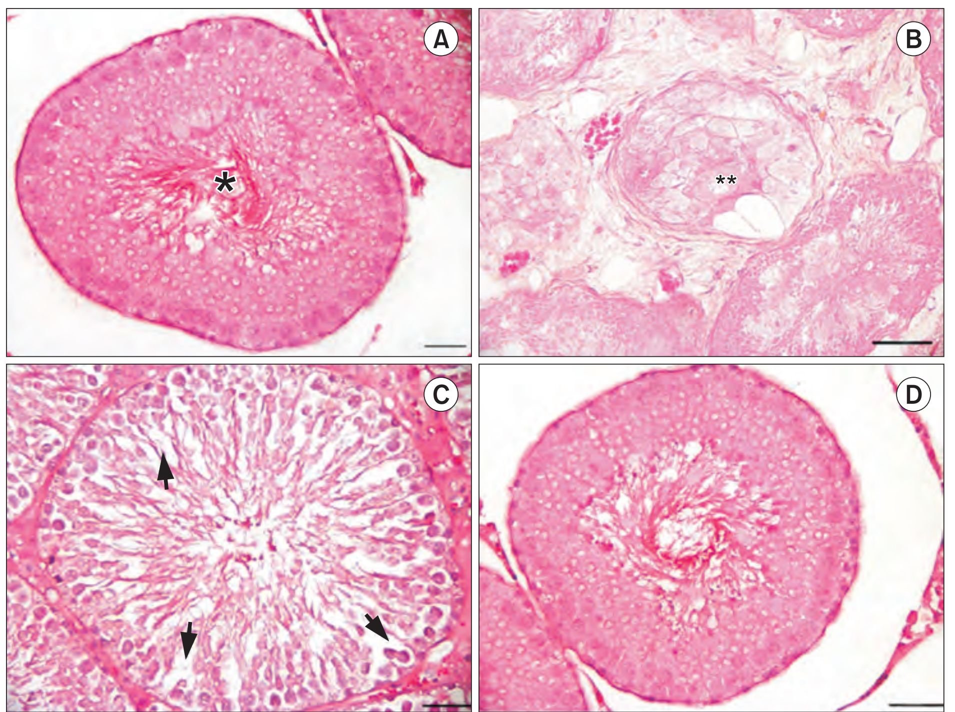
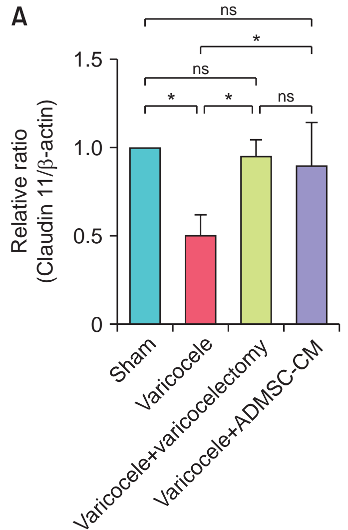


Fig. 6. Histological organization of testes. (A) Sham group: the seminiferous tubules showed normal seminiferous epithelium organization (*). (B) Varicocele group: hyaline degeneration and germ cell disorganization in the seminiferous tubules (**). (C) Varicocele+varicocelectomy: openings in the seminiferous epithelium (black arrows). (D) Varicocele+ADMC-CM: Seminiferous tubule showed normal morphology. The openings in the interstitial area are due to the tissue preparation (H&E, magnification ×400) (bar indicates 50 μm). ADMSC-CM: conditioned medium of adipose-derived mesenchymal stem cell.



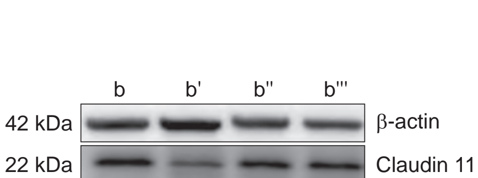


Fig. 7. Comparison of Claudin-11 amounts in the testes of sham (control), varicocele, varicocele+varicocelectomy, and varicocele+ADMSC-CM groups. (B) Representative blots are also shown (b: sham [control], b′: varicocele, b″: varicocele+varicocelectomy, b‴: varicocele+ADMSC-CM). ns: p>0.05, *p≤0.05

cant difference was observed between the two treatment groups (p=0.9657). No significant difference was observed in the Claudin-11 levels between the varicocelectomy (p=0.9765) and ADMSC-CM (p=0.8237) groups and the control group (Fig. 7).

DISCUSSION

ADMSCs can be easily collected in larger quantities with less donor site morbidity, making them preferred in cellular therapies and tissue engineering studies [24]. Although limited in number, there are studies investigating the effect of injection of ADMSCs in the treatment of varicocele [25]. The ability of MSCs to differentiate into osteogenic, adipogenic, and chondrogenic cells raises concerns that MSCs may differentiate into different tissue types in the testicles, causing more harm than benefit. Failure to improve sperm parameters in some patients and potential complications (such as testicular atrophy, hydrocele, and etc.) create controversy about the effectiveness of varicocelectomy, which is considered the gold standard in clinical varicocele

treatment [26].

Considering these limitations, we evaluated the efficacy and safety of ADMSC-CM treatment for varicocele. ADMSC-CM does not include any cells but contains all the paracrine factors secreted by these MSCs, eliminating the concerns about injecting antigenic cells into the testicular tissue, which have the potential to differentiate to other tissues. Varicocele clinically causes low sperm count, decreased sperm motility, and decreased normal sperm morphology [27]. In our study, consistent with the current literature, the varicocele group exhibited significantly lower normal sperm morphology and progressive motility rates compared to the treatment and control groups. Both varicocelectomy and ADMSC-CM treatments were successful in preserving the sperm parameters (concentration, motility, progressive motility, normal morphology) in the rats with varicocele. There was no significant difference in the rates between ADMSC-CM and varicocelectomy treatments, suggesting that these treatments have similar ameliorative effects (Table 2, Fig. 4).

One of the reasons for the negative effects of varicocele on sperm parameters is the increased oxidative stress [28]. MDA levels in the varicocele groups were significantly higher than the control group, and the amounts of SOD, which represents the antioxidant enzyme level, were significantly lower. Varicocelectomy and ADMSC-CM treatments significantly reduced MDA levels, along with a significantly increase in SOD levels in these groups, compared with the varicocele groups. However, both treatment methods failed to reach to the MDA and SOD levels in the sham group. Similar to the results on sperm parameters, no significant differences in SOD and MDA levels were observed between the treatment groups (Table 1, Fig. 3).

Varicocele also changes testicular histology due to its detrimental effects on the testicular parenchyma. In testicular tissue samples obtained from men with varicocele, maturation arrest, germ cell degeneration, atrophy or hyperplasia of Leydig cells, basal membrane thickening, seminiferous tubule lumen narrowing, and degeneration of Sertoli cells were observed [29]. In our experiment, the varicocele group had the lowest Johnsen's Score. Although both treatment groups had better mean Johnsen's Scores compared with the varicocele group, they were still lower than those in the control group. No significant difference was observed in the varicocelectomy and varicocele groups ($p=0.1395$). However, the modest increase in mean Johnsen's Score in the ADMSC-CM group was found to be significantly higher than in the varicocele group ($p=0.0006$) (Table 3, Fig. 6). No significant difference was observed between varicocelectomy and ADMSC-CM groups ($p=0.114$).

The BTB has a very important role in the spermatogenesis process. Claudin-11 is an important tight junction protein expressed by Sertoli cells that plays a role in the formation of BTB, and previous studies have shown that varicocele causes degeneration on BTB by affecting Claudin-11 levels [30]. In our study, consistent with the literature, significantly lower Claudin-11 levels were detected in the varicocele group compared to the control and treatment groups. However, the Claudin-11 levels in the ADMSC-CM and varicocelectomy treatment groups were almost equal to the control group (Fig. 7). BTB disruption due to the significant decrease in Claudin-11 levels in the varicocele group may be closely related to the degeneration of testicular histology.

Several limitations should be acknowledged in our study. Firstly, the use of an animal model was imperative to elucidate the therapeutic effects of ADMSC-CM on varicocele. While animal models provide valuable insights, there are inherent differences between animal physiology and human biology that must be considered when interpreting the results and their translational relevance.

Furthermore, the study's timeframe was limited to four weeks of ADMSC-CM injections following varicocele induction, precluding a comprehensive evaluation of the treatment's long-term stability and enduring effects. Future investigations extending beyond this timeframe would be instrumental in better understanding the durability and sustainability of the observed therapeutic outcomes.

Moreover, a notable limitation of our study is the lack of assessment of the specific types and quantities of proteins present in the ADMSC-CM injections. This limitation arises from the complexity and cost associated with conducting advanced proteomic analyses. While these assessments could provide valuable insights into the molecular mechanisms underlying the therapeutic effects of ADMSC-CM, similar studies in the literature have faced similar challenges due to the technical demands of such analyses [14,15,21].

In summary, while our study provides important insights into the potential of ADMSC-CM as a thera-

peutic intervention for varicocele, it is essential to recognize and address these limitations to inform future research endeavors and clinical applications effectively. Safety, efficacy, optimal administration method and cost-effectiveness of ADMSC-CM treatment must be established and compared with those of conventional varicocelectomy operation. In light of these findings, ADMSC-CM may be considered as effective as varicocelectomy in the treatment of varicocele in improving sperm parameters, ameliorating BTB degeneration and preserving testicular histology. Therefore, ADMSC-CM treatment may have the potential to be an alternative approach for a group of patients with clinical varicocele, who are concerned about the surgical operations.

CONCLUSIONS

To our knowledge, this is the first study to evaluate the therapeutic effects of intratesticular injection of acellular ADMSC-CMs in a surgically induced varicocele model in rats. The therapeutic effect mechanism exhibited by ADMSC-CM may involve secretory products of known anti-inflammatory and regenerative factors of MSCs. ADMSC-CM injection appears to be a promising new strategy in the treatment of varicocele.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: ECS, BK. Data curation: MVB, SK. Formal analysis: BK, MVB. Funding acquisition: IK, RZGO. Investigation: ECS, MVB, BK, CC. Methodology: SK, ECS. Project administration: IK, BK. Resources: IK, MVB. Software: CC, RZGO. Supervision: IK, ECS. Validation: MVB, CC. Visualization: BK, ECS. Writing – original draft: MVB, ECS. Writing – review & editing: ECS, IK.

Data Sharing Statement

The data analyzed for this study have been deposited in HARVARD Dataverse and are available at https://doi.org/10.7910/DVN/ZXGSHG.

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