Bulgarian Journal of Veterinary Medicine, 2026, **29**, No 1, 149–163
ISSN 1311-1477; DOI: 10.15547/bjvm.2024-0126

Original article

THERAPEUTIC EFFECT OF BONE MARROW MESENCHYMAL STEM CELLS FOR TREATMENT OF INDUCED MILD TESTICULAR DEGENERATION IN DOGS

A. A. HAFEEZ[1], M. FATHI[1], A. M. SHAHAT[1], M. M. BAHR[2], A. A. SHAMAA[2] & K. H. EL SHAHAT[1]

[1]Theriogenology Department, Faculty of Veterinary Medicine, Cairo University, Egypt;
[2]Surgery, Anesthesiology and Radiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt

Summary

Hafeez, A. A., M. Fathi, A. M. Shahat, M. M. Bahr, A. A. Shamaa & K. H. El Shahat, 2026. Therapeutic effect of bone marrow mesenchymal stem cells for treatment of induced mild testicular degeneration in dogs. *Bulg. J. Vet. Med.,* **29**, No 1, 149–163.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are extensively used in regenerative medicine. The present work was aimed at investigating the effect of autologous BM-MSCs on semen characteristics and the blood flow of testes associated with hormonal levels of testosterone in testicular degeneration in stray dogs with induced mild heat stress by scrotal insulation. Ten stray male dogs were used in the study, which were exposed to mild heat stress (HS) through padding of the scrotum with a wool-blend sock for 96 h. Then, the dogs (n=10) were randomly distributed into 2 groups; group one (n=5; CON) was injected with 1 mL of phosphate-buffered saline (PBS) once in each testicle and considered as control, whereas group two (n=5; BM-MSCs) was injected intra-testicularly with autologous bone marrow MSCs once for each testicle (1 mL of PBS containing the harvested MSCs) 3 weeks following HS induction. Testicular haemodynamics and semen pictures were determined at weeks 0, 2, 4, 6, 8 and 10 in both groups. The results showed that the dogs treated with autologous BM-MSCs had higher end diastolic velocity (EDV) and and peak systolic velocity (PSV) values ($P<0.05$) between 6^{th} to 10^{th} week following injection than those before treatments. However a significant ($P<0.05$) decrease in resistance index (RI) and pulsatility index (PI) values was also noticed from the 4^{th} until the 10^{th} week. The single intra-testicular injection of autologous BM-MSCs resulted in significantly ($P<0.05$) increased sperm cell concentration, motility %, and live and normal spermatozoa, where the maximum value appeared after 10 weeks post-treatment of dogs as compared to day 0 and to the control dogs. Serum levels of testosterone and nitric oxide (NO) were significantly higher ($P<0.05$) in BM-MSCs-treated group from the 2^{nd} week until the 10^{th} week following injection than those before treatment and in the control group. In conclusion, this study demonstrates the effectiveness of the autologous BM-MSCs treatment in increasing sperm concentration, motility % and also improving testicular blood flow besides levels of testosterone and NO. Further studies are needed to validate these findings.

Key words: autologous BM-MSCs, Doppler indices, nitric oxide, semen evaluation, testosterone

INTRODUCTION

Testicular degeneration (TD) affects between 15% and 58% of male dogs (Câmara *et al.*, 2018), making it one of the most prevalent reasons for acquired infertility and poor semen quality (Fontbonne, 2011). Testicular degeneration is characterised by the loss of testicular function due to structural deterioration of the testis (Turner, 2007). This condition can impact one testis (unilateral) or both testes (bilateral) and may involve the entire testis or just a portion of it. The testis is particularly vulnerable to damage, which can result from either reversible or permanent degeneration of its seminiferous epithelium caused by various factors (Parkinson, 2001). These factors include autoimmune conditions, radiation exposure, excessively high scrotal temperatures, scrotal trauma, exposure to toxins, certain nutritional deficiencies, and infections from pathogenic organisms. Additionally, dogs have been reported to have idiopathic TD, although no underlying cause has been identified for this form (Fontbonne, 2011).

Scrotal insulation, which results in increased testicular temperature, disrupts spermatogenesis (Hochereau-de Reviers *et al.*, 1993). Experimental studies involving scrotal insulation have revealed several adverse effects on ejaculates, including decreased sperm output (Arman *et al.*, 2006), a lower percentage of motile sperm (Arman *et al.*, 2006), reduced integrity of sperm plasma membranes, and a higher incidence of spermatozoa with abnormal morphology (Barth & Bowman, 1994).

Stem cell therapy has emerged as a prominent field in both human and veterinary medicine due to its immunomodulatory effects, ability to proliferate, potential for differentiation, anti-apoptotic properties, and expression of angiogenic cytokines (Kossl *et al.*, 2021). Additionally, various studies have demonstrated the capacity of stem cells to restore testicular structures and functions in multiple models of testicular damage induced in laboratory animals (Ismail *et al.*, 2023b).

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be differentiated into various types of cells such as neuronal cells, chondrocytes, adipocytes, cardiomyocytes and osteoblasts *in vitro* and *in vivo* under controlled conditions (Ishikawa *et al.*, 2006). These cells can be isolated from many tissues, including fat, skin, and even the brain (Jordan *et al.*, 2009). However, the most common source to obtain these cells is the bone marrow.

The bone marrow has traditionally been seen as an organ composed of two main systems: the haematopoietic tissue, which is the primary or main type of blood-forming tissue and the associated supporting stroma. The bone marrow is constituted of two separate and distinct stem cells. The haematopoietic stem cells (HSC) are responsible for the production and maintenance of all mature blood cells. The mesenchymal stem cells (MSC) constitute the bone marrow stroma (Bonnet, 2003). Due to their multilineage differentiating ability, MSCs expand the possibilities of regenerative medicine (Pezzanite *et al.*, 2015), where their application helps to replace bone tissue and cartilage (Kangari *et al.*, 2020). However, the low survival and transient retention of transplanted MSCs in host tissue (Miao *et al.*, 2017) indicate that MSCs exert their therapeutic effects via secretion of bioactive factors that provide a favourable microenvironment to facilitate the repair and regeneration of injured tissues (Caplan, 2017).

The paracrine effect of MSCs plays an important role, as angiogenesis, neuropro-

tection, and immunoregulation in the target tissue are affected through the production of important growth and trophic factors or bioactive molecules (Han *et al.*, 2022). Therefore, this study posits that the intratesticular administration of bone marrow mesenchymal stem cells may help to improve the low semen quality caused by heat stress in dogs.

MATERIALS AND METHODS

Experimental location and ethical approval

This study was conducted at the Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University (latitude 30°01' N; longitude 31°21' E), from April 2024 to July 2024. The study received ethical approval from the institutional animal care committee of the Faculty of Veterinary Medicine, Cairo University (Approval Vet CU 18042024892)

Animals and management

Ten stray male dogs (average body weight: 30±0.5 kg; age: 2±0.5 years) of unknown breeding history were included in this study. Prior to the initiation of the experiment, all males underwent a clinical examination, followed by ultrasonography evaluation of the male reproductive organs, including the testes, epididymis, and prostate. All findings were normal in terms of texture, size, and echogenicity. Ultrasonography examination, blood collection, as well as semen collection and evaluation, were conducted on dogs for two consecutive weeks as a control. The results of the semen analysis indicated a concentration of 295.6±1.36 million spermatozoa per milliliter ($\times 10^6$/mL), motility percentage of 76.6±0.92%, live/dead ratio of 91.4±0.92 and abnormalities percentage of 12±0.70%.

All dogs were exposed to mild heat stress (HS) through padding of the scrotum with a wool-blend sock for 96 h (Shahat *et al.*, 2021). Following their exposure to this HS, weekly examinations to monitor the effect of the HS on the semen quality were conducted. Three weeks after HS induction semen analysis results indicated a spermatozoa concentration of 128±0.70 $\times 10^6$/mL, motility percentage of 25.00±0.70%, live/dead ratio 70.00±0.70 and abnormalities percentage of 74.20± 0.86%. Then, the dogs (n=10) were randomly distributed into 2 groups; group one (n=5; CON) was injected with 1 mL of phosphate-buffered saline (PBS) once in each testicle and considered a control, whereas group two (n=5; autologous BM-MSCs) was intra-testicularly injected with autologous bone marrow MSCs once for each testicle (1 mL of PBS containing the harvested MSCs) 3 weeks following HS induction.

All dogs were examined every 2 weeks for the following 10 weeks post injection. All dogs in the study were kept indoors, given regular exercise, and provided with a commercial diet consisting of cereals such as rice bran, lipids, vegetables, and vitamins. They also had unlimited access to water.

Experimental design

Males were subjected to routine examination at weeks 0, 2, 4, 6, 8 and 10 to determine the effect of the single intratesticular injection of autologous BM-MSCs on testicular volume, serum testosterone and nitric oxide levels and semen pictures compared to control animals. Dogs were subjected to blood collection, semen collection and ultrasonography examination. The assessment was per-

formed on week 0 before injection, and the examination was extended to 10 weeks.

Bone marrow MSC isolation and identification

Fresh bone marrow (BM) was aspirated from the proximal tibia of healthy dogs under general anaesthesia. The anaesthesia protocol included premedication with subcutaneous atropine sulfate (0.1 mg/kg), intramuscular xylazine (1 mg/kg), and intravenous induction with ketamine (10 mg/kg) (Bahr *et al.*, 2021). Bone marrow was obtained from the proximal ends of both tibias using an 18-gauge bone marrow biopsy needle connected to a heparinised 20-mL collection syringe. After securing the bone marrow needle in place within the tibia, the mandrel was removed, and 20 mL of bone marrow was aspirated from each tibia (Papa *et al.*, 2024). To isolate BM-MSCs, a density gradient was performed using Ficoll solution (Sigma-Aldrich). The collected MSCs were then suspended in phosphate-buffered saline (PBS) (Biowest) (Jung *et al.*, 2008). Approximately 1 mL contains 1×10^6 MSCs (Yoon *et al.*, 2012; Papa *et al.*, 2024).

Cells were identified as MSCs based on their morphology under inverted light microscope (Jung *et al.*, 2008).

Intra-testicular injection of BM-MSCs

BM-MSCs was administered to the dogs, which were placed under a general anaesthesia regimen that included subcutaneous remedication with atropine sulfate (0.1 mg/kg), intramuscular xylazine (1 mg/kg), and intravenous induction with ketamine (10 mg/kg) (Silva *et al.*, 2020). Prior to the injection, the scrotum was clipped and disinfected with betadine.

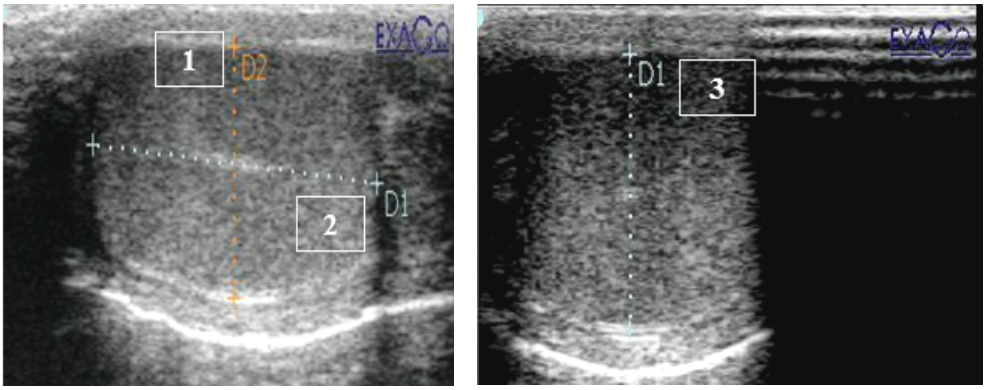
Injections were performed using a sterile 21-gauge needle inserted at the proximal end and withdrawn to the distal end, allowing for linear infiltration of the EV solution along the entire length of the needle track. The needle was positioned from the caudoventral aspect of each testis, approximately 1 cm from the epididymal tail, and directed toward the dorsocranial aspect of the testis (Jana & Samanta, 2007). One mL of PBS-MSCs suspension was injected into each testicle once.

Ultrasonography evaluation

Ultrasound examinations on the right and left testes of each dog were conducted using the EXAGO Doppler ultrasound device (EXAGO, IMV, France) equipped with a 7.5 MHz linear array transducer. The dogs were positioned in dorsal recumbency, and acoustic gel was applied to the skin before placing the transducer on the lateral surface of the testis. Testicular length and width were measured using longitudinal and transverse B-mode imaging, with the mediastinum serving as a reference point. Testicular volume was calculated using the formula for an ellipsoid: V=length×width×height×0.5236 (de Souza *et al.*, 2015) as shown in Fig. 1.

Measurement of the testicular artery Doppler parameters

For measuring testicular artery flow, color Doppler ultrasound was employed with the transducer initially placed at the neck of the scrotum to locate the tortuous distal (looping) segment of the supratesticular artery, just cranial to the cranial pole of the testis. The colour gain was adjusted to minimise excess colour noise, and the pulse-wave Doppler gate was positioned within the vessel lumen. Three cardiac cycles were used to obtain mean values for peak systolic velocity (PSV) and end-diastolic velocity (EDV), which was then



A **B**

Fig. 1. Ultrasonography revealed the testicular dimensions to estimate the testicular volume using the ellipsoid shape formula. Length (1) and height (2) are measured in the longitudinal scan (**A**); while the testicular width (3) is measured in the transverse scan (**B**).

utilised by the machine's software to calculate the resistance index (RI) and pulsatility index (PI). The operator and machine settings (depth: 4.5–5.5 cm; pulse repetition frequency: 2.5 kHz; wall filter: 5 cm/s; sample gate: 2.0 mm) were kept consistent across all examinations. The angle between the Doppler beam and the vessel's long axis was maintained at less than 60°, with angle corrections applied as necessary. In most instances, an angle of 0° was used (de Souza *et al.*, 2015).

Semen collection and evaluation

The semen collection procedure was ideally conducted with an estrous bitch present. Initially, the dog's penis was vigorously stimulated through the prepuce at the level of the bulbus glandis until a partial erection was achieved. Subsequently, the penis was squeezed between the thumb and index finger while pelvic thrusting was employed to induce full erection. Following the collection of the first two fractions, the semen samples were sent to the laboratory for further analysis. The ejaculates were collected into a cup that had been preheated to 36–38 °C (Mickelsen *et al.*, 1993). The first two fractions were analysed for sperm motility, morphology, live/dead ratio, and concentration. Sperm motility was assessed by placing a drop of semen on a warmed microscope slide and estimating the progressive movement of sperm to the nearest 5% using a light microscope (100× and 400× magnification) (LABOMED, Labo America Inc., U.S.A.).

To evaluate the numbers of live and dead spermatozoa as well as sperm morphology, a small drop of semen was placed on a warmed slide and stained with eosin-nigrosin. A total of 100 to 200 spermatozoa were counted under oil immersion at 1000× magnification (Mickelsen *et al.* 1993). Sperm concentration was measured using a haemocytometer (Neubauer counting chamber) and a light microscope at 400× magnification (LABOMED, Labo America Inc., U.S.A.) (England, 1999).

Blood sampling and analysis

Two milliliters of blood were collected from the cephalic vein and the samples were centrifuged at 3,000 g for 5 min at

room temperature to obtain serum (Domosławska & Zdunczyk, 2020). The resulting serum samples were then stored at −20 °C until further hormonal and biochemical analyses were conducted.

Serum testosterone levels were measured using commercial ELISA kits (DiaSino Laboratories Co., Ltd., Zhengzhou, China) in accordance with the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 3.3% and 4.8%, respectively, with an assay sensitivity of 0.05 ng/mL. Additionally, nitric oxide (NO) levels in the serum samples were assessed using a spectrophotometer (Prietest Touch, Robonik, India) at 540 nm, employing commercial nitric oxide kits (Bio-diagnostic, Dokki, Egypt) as per the manufacturer's guidelines.

RESULTS

All animals tolerated the intra-testicular injections without adverse effects. There were no instances of fever or significant inflammatory swelling of the testis. Food consumption remained unaffected across the two groups of animals throughout the experiment. All dogs that received bone marrow MSCs remained in good health during the experimental period. No notable behavioural changes were observed in the animals throughout the study.

Testicular volume

Table 1 describes the influence of the single intra-testicular autologous BM-MSCs injection on the testicular volume. At the beginning of the experiment, no significant change in testicular volume could be detected among the two groups. Furthermore, the single intra-testicular injection of BM-MSCs resulted in non-significant increase ($P>0.05$) in testicular volume when compared to the control group all over the experimental study (70 days), respectively.

Testicular blood flow

The data in Table 2 exhibit changes in testicular blood flow. At the beginning of the experiment, no significant between-groups change in testicular blood flow could be detected. It was clear that a single intra testicular injection of the autologous BM-MSCs resulted in a significant ($P<0.05$) decrease in RI and PI values compared to control untreated group at the 4th week until the 10th week of the study. With respect to duration of study, a significant ($P<0.05$) decrease in RI and PI values was also noticed from the 2nd week until the 10th week when compared with those obtained at day 0. However, the dogs treated with autologous BM-MSCs had higher EDV and PSV ($P<0.05$) from the 6th till the 10th week following injec-

Table 1. Effects of single intra-testicular injection of autologous bone marrow mesenchymal stem cells (BM-MSCs) on testicular volume (cm^3) in induced mild testicular degeneration in stray male dogs (mean ± SEM)

Study intervals	Control group (n=5)		BM-MSCs group (n=5)	
	Right testis	Left testis	Right testis	Left testis
0 week	7.23±0.14	6.79±0.24	7.14±0.08	6.98±0.35
2nd week	7.05±0.18	6.90±0.36	7.32±0.26	6.99±0.36
4th week	7.10±0.15	6.72±0.34	7.49±0.23	7.19±0.22
6th week	7.09±0.16	6.85±0.13	7.50±0.25	7.10±0.29
8th week	7.21±0.14	6.91±0.38	7.64±0.33	7.83±0.15
10th week	7.24±0.16	6.90±0.35	7.80±0.26	7.84±0.16

Table 2. Effects of a single intra-testicular injection of autologous bone marrow mesenchymal stem cells (BM-MSCs) on the testicular blood flow in stray male dogs with induced mild testicular degeneration (mean± SEM)

Study intervals	Parameter	Control group (n=5)	BM-MSCs group (n=5)
0 week	Peak systolic velocity (cm/s)	21.04±0.53$^{A, a}$	21.64±0.64$^{A, a}$
	End-diastolic velocity (cm/s)	5.37±0.20 $^{A, a}$	5.77±0.3 $^{A, a}$
	Resistance index	0.74±0.02 $^{A, a}$	0.75±0.07 $^{A, a}$
	Pulsatility index	1.50±0.05 $^{A, a}$	1.50±0.05 $^{A, a}$
2nd week	Peak systolic velocity (cm/s)	22.24±0.61 $^{A, a}$	22.44±0.70 $^{A, a, b}$
	End-diastolic velocity (cm/s)	6.15±0.43 $^{A, a}$	6.35±0.26 $^{A, a}$
	Resistance index	0.71±0.01 $^{A, a}$	0.70±0.03 $^{A, b}$
	Pulsatility index	1.37±0.01 $^{A, b}$	1.37±0.06 $^{A, b}$
4th week	Peak systolic velocity (cm/s)	20.84±0.91 $^{A, a}$	23.64±1.19 $^{A, a, b}$
	End-diastolic velocity (cm/s)	5.39±0.55 $^{A, a}$	6.39±0.55 $^{A, a}$
	Resistance index	0.72±0.01 $^{A, a}$	0.64±0.01 $^{B, c}$
	Pulsatility index	1.36±0.08 $^{A, b}$	1.16±0.02 $^{B, c}$
6th week	Peak systolic velocity (cm/s)	21.00±1.07 $^{A, a}$	24.10±0.51 $^{B, a, b}$
	End-diastolic velocity (cm/s)	5.38±0.55 $^{A, a}$	8.26±0.45 $^{B, b}$
	Resistance index	0.73±0.07 $^{A, a}$	0.61±0.05 $^{B, c, d}$
	Pulsatility index	1.37±0.07 $^{A, b}$	1.09±0.01 $^{B, c, d}$
8th week	Peak systolic velocity (cm/s)	20.52±0.99 $^{A, a}$	24.84±0.45 $^{B, b}$
	End-diastolic velocity (cm/s)	5.38±0.55 $^{A, a}$	8.67±0.62 $^{B, b}$
	Resistance index	0.72±0.08 $^{A, a}$	0.59±0.01 $^{B, d}$
	Pulsatility index	1.36±0.09 $^{A, b}$	1.00±0.04 $^{B, d, e}$
10th week	Peak systolic velocity (cm/s)	20.54±0.98 $^{A, a}$	24.86±0.44 $^{B, b}$
	End-diastolic velocity (cm/s)	5.38±0.55 $^{A, a}$	8.87±0.51 $^{B, b}$
	Resistance index	0.73±0.07 $^{A, a}$	0.59±0.01 $^{B, d}$
	Pulsatility index	1.36±0.08 $^{A, b}$	0.98±0.03 $^{B, e}$

Means with different superscripts (A,B) within the same row and different superscripts (a,b,c,d,e) within the same column were significantly different at P<0.05.

tion than those in control untreated group (Fig. 2 and 3).

Analysis of semen parameters

The effect of intra-testicular injection of BM-MSCs on semen characteristics in dogs is shown in Table 3. The sperm cell concentration in BM-MSCs treated dogs was significantly higher (P<0.05) at 2 weeks till the end of the study (10 weeks) than those seen before treatments and in the control group. The maximum value of sperm cell concentration obtained from treated dogs was attained by the 10th week following injection. No between-group differences in sperm motility values were detected at the start of experiment (day 0) till the 6th week. On the other hand, autologous BM-MSCs-treated dogs showed a substantial increase in sperm motility from the 8th till the 10th weeks following injection when compared to the control group. Live/dead spermatozoa (L/D) ratio increased significantly (P<0.05) from the 4th week, and continued to attain the maximum value at the 10th week after the BM-MSCs treatment vs control dogs. The reverse trend was obtained in sperm cell abnormality. The intra-testicular BM-

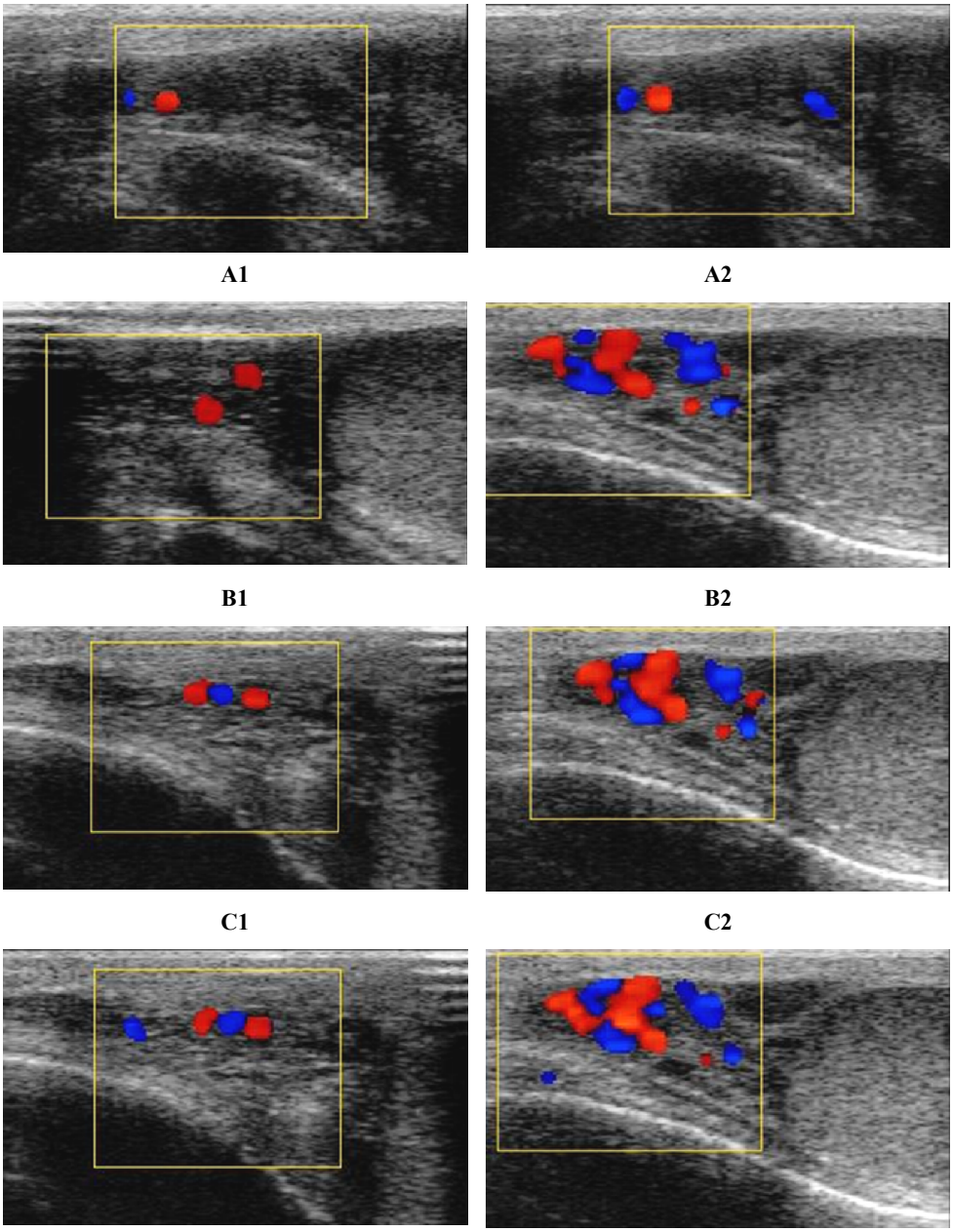


Fig. 2. Colour Doppler ultrasonography images of the testes before and after injection. **A1**: 0 week in the control group. **B1, C1,** and **D1**: 2 weeks, 4 weeks, and 6 weeks, respectively, in the control group. **A2**: 0 week in the bone marrow MSCs group before the injection. **B2, C2,** and **D2**: 2 weeks, 4 weeks, and 6 weeks, respectively, in the BM-MSCs group post-injection.

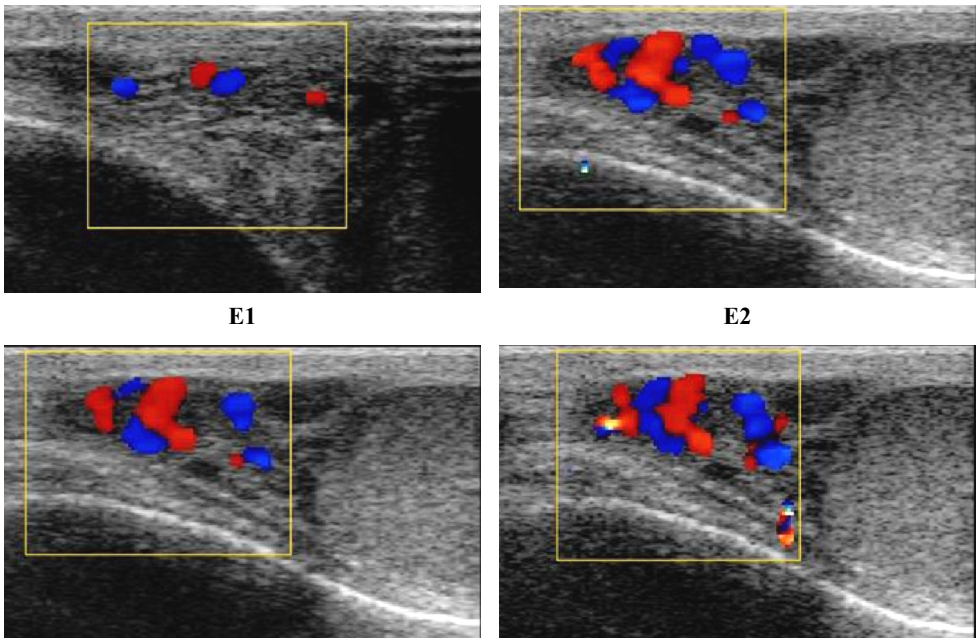


Fig. 2 (cont'd). Colour Doppler ultrasonography images of the testes before and after injection. **E1** and **F1**: 8 weeks and 10 weeks, respectively, in the control group. **E2** and **F2**: 8 weeks and 10 weeks, respectively, in the BM-MSCs group post-injection.

MSCs injection in dogs increased significant ($P<0.05$) sperm cell concentration, motility, and live spermatozoa compared to those in controls.

Serum testosterone and NO levels

All autologous BM-MSCs treated dogs had significantly higher testosterone and NO levels ($P<0.05$) at the 2nd week to the 10th week following injection compared to day 0 and to the control group. The maximum testosterone and NO levels in BM-MSCs treated dogs were observed at the 8th and the 10th weeks post injections. In addition, the autologous BM-MSCs dogs showed higher testosterone values ($P<0.05$) from the 2th to the 10th week vs day 0 (Table 4). However NO levels showed higher values ($P<0.05$) from the 4th to the 10th week than those obtained before treatment (Table 5).

DISCUSSION

The study aimed to evaluate the effect of a single intra-testicular autologous BM-MSCs injection in testicular degeneration induced through mild heat stress by scrotal insulation in stray dogs. Induced hyperthermia has been employed as a model to induce acute testicular degeneration across several species (Henning *et al.*, 2014). Testicular degeneration can be triggered by various chemical, physical, and microbial agents and is marked by the disruption of testicular structure and the loss of functionality (Fontbonne, 2011). In the current study, testicular heat stress led to a reduction in sperm concentration and

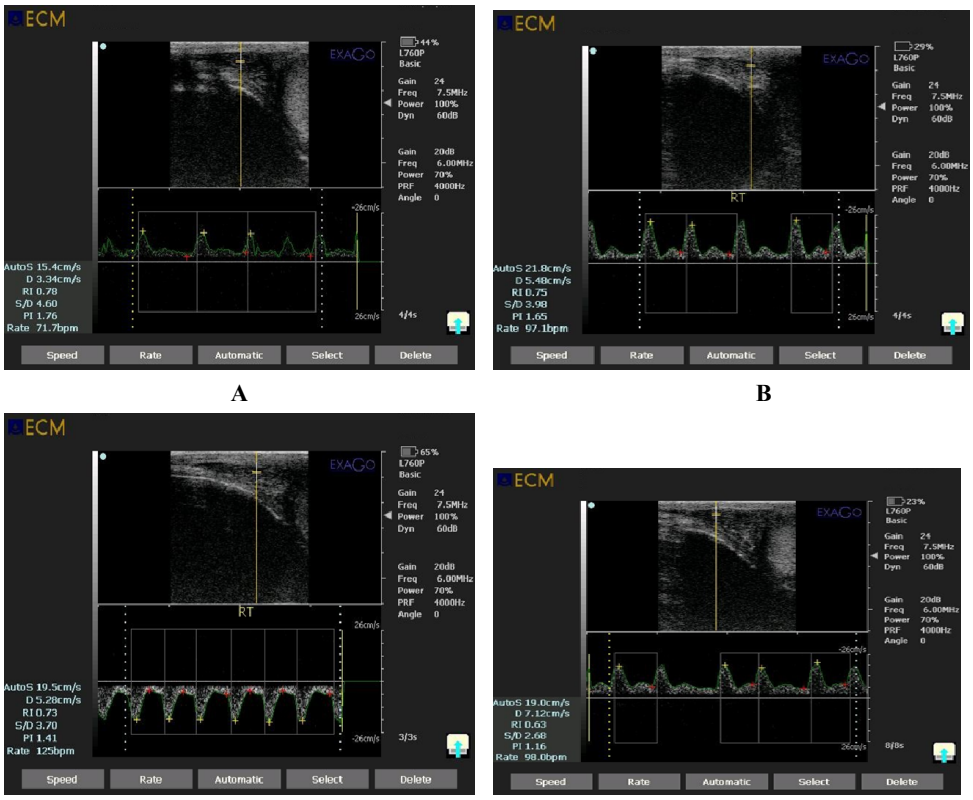


Fig. 3. Pulsed-wave Doppler ultrasonography images of the testicular artery. **A**: control group and **B**: BM-MSCs group at 0 week before the injection. **C**: control group and **D**: BM-MSCs group 10 weeks post injection with the automatic calculation of both Doppler indices (RI and PI). The yellow star showed the maximum systolic point of velocity (MSV; cm/sec), while the red star showed the end point of velocity due to relaxation (EDV; cm/sec). RI=resistance index, PI=pulsatility index.

motility, as well as an increased number of dead and abnormal sperm. Previous research in canines has shown that short-term scrotal hyperthermia can lead to a considerable decline in semen quality (Henning *et al.*, 2014). However, it is noteworthy that although semen parameters recovered over time in all the dogs in the present study, the dogs treated with autologous BM-MSCs exhibited improved semen quality compared to the control group, indicating a potential therapeutic benefit of MSCs in protecting testicular germ cells.

Results revealed a statistically significant ($P<0.05$) improvement in sperm concentration, motility, and live/dead ratios from weeks 2 to 10 post-injection compared to both control group and pre-treatment levels. This rapid improvement corresponds with the 62-day spermatogenesis cycle in stray dogs (Soares *et al.*, 2009). Treated dogs also exhibited significantly higher levels of NO and testosterone ($P<0.05$) compared to controls and

Table 3. Effects of a single intra-testicular injection of autologous BM-MSCs on sperm cell concentration ($\times 10^6$/mL), motility percentage, live/dead ratio, and abnormalities percentage in stray male dogs with induced mild testicular degeneration (mean ± SEM)

Study intervals	Parameter	Control group (n=5)	BM-MSCs group (n=5)
0 week	Sperm cell concentration ($\times 10^6$/mL)	129.14±0.51 ^{A,a}	128.00±0.70 ^{A,a}
	Motility percentage	25.80±0.86 ^{A,a}	25.00±0.70 ^{A,a}
	Live/dead ratio	70.60±0.92 ^{A,a}	70.00±0.70 ^{A,a}
	Abnormalities percentage	73.00±0.70 ^{A,a}	74.20±0.86 ^{A,a}
2nd week	Sperm cell concentration ($\times 10^6$/mL)	127.00±0.70 ^{A,a,b}	131.12±0.78 ^{B,b}
	Motility percentage	45.60±0.92 ^{A,b}	45.00±0.71 ^{A,b}
	Live/dead ratio	66.40±0.92 ^{A,b}	66.00±0.70 ^{A,b}
	Abnormalities percentage	75.60±0.92 ^{A,a}	76.00±0.70 ^{A,a}
4th week	Sperm cell concentration ($\times 10^6$/mL)	126.08±0.66 ^{A,b}	133.18±0.70 ^{B,b}
	Motility percentage	43.20±0.86 ^{A,c}	45.00±0.70 ^{A,b}
	Live/dead ratio	69.00±0.70 ^{A,a,b,c}	74.80±0.86 ^{B,c}
	Abnormalities percentage	75.00±0.70 ^{A,a,b}	65.60±0.92 ^{B,b}
6th week	Sperm cell concentration ($\times 10^6$/mL)	125.28±0.75 ^{A,b}	137.30±0.66 ^{B,c}
	Motility percentage	42.40±0.92 ^{A,c}	45.00±0.71 ^{A,b}
	Live/dead ratio	70.00±0.71 ^{A,c}	81.20±0.87 ^{B,d}
	Abnormalities percentage	77.00±0.71 ^{A,b}	61.20±0.86 ^{B,c}
8th week	Sperm cell concentration ($\times 10^6$/mL)	135.12±0.62 ^{A,c}	150.18±0.80 ^{B,d}
	Motility percentage	45.80±0.86 ^{A,b}	51.60±0.92 ^{B,c}
	Live/dead ratio	62.00±0.72 ^{A,d}	91.20±0.86 ^{B,e}
	Abnormalities percentage	41.20±0.86 ^{A,c}	25.00±0.70 ^{B,d}
10th week	Sperm cell concentration ($\times 10^6$/mL)	135.10±0.64 ^{A,c}	154.94±0.67 ^{B,e}
	Motility percentage	44.80±1.06 ^{A,b,c}	56.00±0.70 ^{B,d}
	Live/dead ratio	66.00±0.70 ^{A,e}	92.20±1.28 ^{B,e}
	Abnormalities percentage	45.20±0.86 ^{A,d}	23.60±1.02 ^{B,d}

Means with different superscripts (A,B) within the same row and different superscripts (a,b,c,d,e) within the same column were significantly different at $P<0.05$.

Table 4. Effects of a single intra-testicular injection of autologous Bone marrow mesenchymal stem cells (BM-MSCs) on serum testosterone (ng/mL) in stray male dogs with induced mild testicular degeneration (mean ± SEM; n=5)

Study intervals	Control group (n=5)	BM-MSCs group (n=5)
0 week	2.22±0.07 ^{A,a}	2.26±0.05 ^{A,a}
2nd week	2.18±0.05 ^{A,a}	2.84±0.09 ^{B,b}
4th week	2.12±0.05 ^{A,a}	3.22±0.10 ^{B,c}
6th week	2.06±0.04 ^{A,a}	3.44±0.08 ^{B,c}
8th week	2.05±0.04 ^{A,a}	4.08±0.07 ^{B,d}
10th week	2.07±0.03 ^{A,a}	4.54±0.09 ^{B,e}

Means with different superscripts (A,B) within the same row and different superscripts (a,b,c,d,e) within the same column were significantly different at $P<0.05$.

Table 5. Effects of a single intra-testicular injection of autologous Bone marrow mesenchymal stem cells (BM-MSCs) on serum nitric oxide (µmol/L) in stray male dogs with induced mild testicular degeneration (mean ± SEM)

Study intervals	Control group (n=5)	BM-MSCs group (n=5)
0 week	$32.77\pm0.42^{A,a}$	$32.91\pm0.30^{A,a}$
2nd week	$31.57\pm0.35^{A,a}$	$33.71\pm0.20^{B,a}$
4th week	$32.64\pm0.29^{A,a}$	$39.42\pm0.28^{B,b}$
6th week	$38.80\pm0.27^{A,b}$	$44.31\pm0.26^{B,c}$
8th week	$43.55\pm0.69^{A,c}$	$58.14\pm0.33^{B,d}$
10th week	$41.75\pm0.54^{A,d}$	$62.49\pm0.64^{B,e}$

Means with different superscripts (A,B) within the same row and different superscripts (a,b,c,d,e) within the same column were significantly different at $P<0.05$.

baseline measurements. Testosterone, essential for maintaining seminiferous tubule structure and function (Jegou & Sharpe, 1993), is directly associated with improved semen quality in treated dogs. NO hinders platelet aggregation and prevents platelet adhesion with endothelial cells, thus playing a direct role in the initiation of the vasodilation process in the reproductive system, as previously described in dogs (Salama *et al.*, 2022).

The specific mechanisms through which MSCs promote regeneration remain unclear. In cases of TD, it is suggested that MSCs facilitate regeneration by releasing growth factors and cytokines that stimulate spermatogenic cell proliferation, provide antioxidant and antiapoptotic effects, and support the functionality of Leydig cells (Ismail *et al.*, 2023a). Intratesticular MSC therapy has been associated with improvements in histological testicular features, such as seminiferous tubule diameter, the height of the germinal epithelial lining, and sperm count as well as enhanced antioxidant capacity, elevated testosterone levels, and better sperm parameters (Ismail *et al.*, 2023b). Notably, in studies of testicular torsion-induced TD in rats, intra-testicular injections of allogeneic MSCs led to significant improvement in testosterone levels, alleviated local oxidative stress, and inhibition of apoptosis in testicular tissue, while also improving sperm function (Sharifian *et al.*, 2022).

Previous studies have demonstrated the potential of MSCs in treating male infertility. Research by Zhang *et al.* (2014) demonstrated restored fertility in azoospermic rats following MSC transplantation. Similarly, adipose-derived mesenchymal stem cells (ADMSCs) treatment repaired damaged testicular tissue, enhanced biochemical parameters, and modified pathological changes caused by cisplatin (Ismail *et al.*, 2023b). These findings highlight the regenerative potential of MSCs in addressing infertility by differentiating into sperm-like cells and supporting spermatogenesis.

In this study, BM-MSCs were shown to enhance testicular blood flow by increasing PSV and EDV while reducing PI and RI in testicular arteries. A previous study demonstrated that BM-MSCs injection produced a higher amount of vascular endothelial growth factor (VEGF), the transforming growth factor b-I (TGFb-I), and NO, all of which contribute to angiogenesis and arteriogenesis, so these changes suggest improved vascular perfusion, facilitating better oxygen and nutrient delivery to the testes (Jin *et al.*, 2012).

Further studies are needed to elucidate the molecular basis of improvement of semen quality and testicular hemodynamics following a single intra-testicular injection of autologous BM-MSCs.

CONCLUSION

This study represents the first report of a single intra-testicular injection of autologous BM-MSCs for treating testicular degeneration in stray dogs through scrotal insulation induced mild heat stress. The findings demonstrate significant improvements in testicular vascularisation, semen quality, and serum testosterone and nitric oxide. These results indicate that BM-MSCs could provide a novel, minimally invasive therapeutic option for enhancing reproductive performance in male dogs and potentially in other species.

REFERENCES

Arman, C., P. Quintana Casares, L. G. Sanchez-Partida & B. Setchell, 2006. Ram sperm motility after intermittent scrotal insulation evaluated by manual and computer‐assisted methods. *Asian Journal of Andrology,* **8**, 411–418.

Bahr, M. M., M. S. Amer, K. Abo-EL-Sooud, A. N. Abdallah, G. G. Shehab & O. S. El-Tookhy, 2021. Proficiency of carboxymethylcellulose as a cryoprotectant. clinical and histological evaluation of cryopreserved heterogenous mesenchymal stem cell-exosomal hydrogel on critical size skin wounds in dogs. *International Journal of Hematology-Oncology and Stem Cell Research,* **15**, 178.

Barth, A. D. & P. A. Bowman, 1994. The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. *The Canadian Veterinary Journal,* **35**, 93.

Bonnet, D., 2003. Biology of human bone marrow stem cells. *Clinical and Experimental Medicine,* **3**, 140–149.

Câmara, L., D. Câmara, F. Maiorino, V. S. Júnior & M. Guerra, 2018. Canine testicular disorders and their influence on sperm morphology. *Animal Reproduction,* **11**, 32–36.

Caplan, A. I., 2017. Mesenchymal stem cells: Time to change the name! *Stem Cells Translational Medicine,* **6**, 1445–1451.

de Souza, M. B., G. C. England, A. C. Mota Filho, C. L. Ackermann, C. V. S. Sousa, G. G. de Carvalho & E. Oba, 2015. Semen quality, testicular B-mode and Doppler ultrasound, and serum testosterone concentrations in dogs with established infertility. *Theriogenology,* **84**, 805–810.

Domosławska, A. & S. Zdunczyk, 2020. Clinical and spermatological findings in male dogs with acquired infertility: A retrospective analysis. *Andrologia,* **52**, e13802.

England, G., 1999. Semen quality in dogs and the influence of a short-interval second ejaculation. *Theriogenology*, **52**, 981–986.

Fontbonne, A., 2011. Infertility in male dogs: Recent advances. *Revista Brasileira de Reprodução Animal,* **35**, 266–273.

Han, Y., J. Yang, J. Fang, Y. Zhou, E. Candi, J. Wang & Y. Shi, 2022. The secretion profile of mesenchymal stem cells and potential applications in treating human diseases. *Signal Transduction and Targeted Therapy,* **7**, 92.

Henning, H., C. Masal, A. Herr, K. Wolf, C. Urhausen, A. Beineke & A. R. Günzel‐Apel, 2014. Effect of short‐term scrotal hyperthermia on spermatological parameters, testicular blood flow and gonadal tissue in dogs. *Reproduction in Domestic Animals,* **49**, 145–157.

Hochereau-de Reviers, M., A. Locatelli, C. Perreau, C. Pisselet & B. Setchell, 1993. Effects of a single brief period of moderate heating of the testes on seminiferous tubules in hypophysectomized rams treated

with pituitary extract. *Reproduction,* **97**, 381–387.

Ishikawa, F., H. Shimazu, L. D. Shultz, M. Fukata, R. Nakamura, B. Lyons & K.I. Nakamura, 2006. Purified human hematopoietic stem cells contribute to the generation of cardiomyocytes through cell fusion. *FASEB Journal,* **20**, 950–952.

Ismail, H. Y., S. Hussein, N. A. Shaker, H. Rizk & Y. Wally, 2023a. Stem cell treatment trials for regeneration of testicular tissue in laboratory animals. *Reproductive Sciences,* **30**, 1770–1781.

Ismail, H. Y., N. A. Shaker, S. Hussein, A. Tohamy, M. Fathi, H. Rizk & Y. Wally, 2023b. Cisplatin-induced azoospermia and testicular damage ameliorated by adipose-derived mesenchymal stem cells. *Biological Research,* **56**, 2.

Jana, K. & P. K. Samanta, 2007. Sterilization of male stray dogs with a single intratesticular injection of calcium chloride: A dose-dependent study. *Contraception,* **75**, 390–400.

Jegou, B. & R. Sharpe, 1993. Paracrine mechanisms in testicular control. In: *Molecular Biology of the Male Reproductive System,* Academic Press, Inc., pp. 271–310.

Jin, H., B. Xia, N. Yu, B. He, Y. Shen, L. Xiao & P. Tong, 2012. The effects of autologous bone marrow mesenchymal stem cell arterial perfusion on vascular repair and angiogenesis in osteonecrosis of the femoral head in dogs. *International Orthopaedics,* **36**, 2589–2596.

Jordan, P. M., L. D. Ojeda, J. R. Thonhoff, J. Gao, D. Boehning, Y. Yu & P. Wu, 2009. Generation of spinal motor neurons from human fetal brain-derived neural stem cells: role of basic fibroblast growth factor. *Journal of Neuroscience Research,* **87**, 318–332.

Jung, D.-I., J.-I. Ha, J.-W. Kim, B.-T. Kang, J.-H. Yoo, C. Park & H.-M. Park, 2008. Canine mesenchymal stem cells derived from bone marrow: isolation, characterization, multidifferentiation, and neurotrophic factor expression in vitro. *Journal of Veterinary Clinics,* **25**, 458–465.

Kangari, P., T. Talaei-Khozani, I. Razeghian-Jahromi & M. Razmkhah, 2020. Mesenchymal stem cells: Amazing remedies for bone and cartilage defects. *Stem Cell Research & Therapy,* **11**, 492.

Kossl, J., P. Bohacova, B. Hermankova, E. Javorkova, A. Zajicova & V. Holan, 2021. Antiapoptotic properties of mesenchymal stem cells in a mouse model of corneal inflammation. *Stem Cells and Development,* **30**, 418–427.

Miao, C., M. Lei, W. Hu, S. Han & Q. Wang, 2017. A brief review: The therapeutic potential of bone marrow mesenchymal stem cells in myocardial infarction. *Stem Cell Research & Therapy,* **8**, 242.

Mickelsen, W., M. Memon, P. Anderson & D. Freeman, 1993. The relationship of semen quality to pregnancy rate and litter size following artificial insemination in the bitch. *Theriogenology,* **39**, 553–560.

Papa, P. M., L. G. Segabinazzi, C. E. Fonseca-Alves, F. O. Papa & M. A. Alvarenga, 2024. Intratesticular transplantation of allogenic mesenchymal stem cells mitigates testicular destruction after induced heat stress in Miniature-horse stallions. *Journal of Equine Veterinary Science,* **132**, 104961.

Parkinson, T., 2001. Fertility and infertility in male animals. In: *Arthur's Veterinary Reproduction and Obstetrics,* Harcourt Publishers Ltd, pp. 695–750.

Pezzanite, L. M., L. A. Fortier, D. F. Antczak, J. M. Cassano, M. M. Brosnahan, D. Miller & L. V. Schnabel, 2015. Equine allogeneic bone marrow-derived mesenchymal stromal cells elicit antibody responses *in vivo. Stem Cell Research & Therapy,* **6**, 54.

Salama, A., E. A. Abdelnaby, I. A. Emam & M. Fathi, 2022. Single melatonin injection enhances the testicular artery hemodynamic, reproductive hormones, and semen parameters in German shepherd dogs. *BMC Veterinary Research,* **18**, 403.

Shahat, A. M., J. C. Thundathil & J. P. Kastelic, 2021. Scrotal subcutaneous temperature is increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decrease sperm quality in bulls and rams. *Journal of Thermal Biology*, **100**, 103064.

Sharifian, P., S. Yari, P. Hasanein & Y. Manteghi Nezhad, 2022. Conditioned medium of bone marrow mesenchymal stem cells improves sperm parameters and reduces histological alteration in rat testicular ischaemia/reperfusion model. *Andrologia,* **54**, e14624.

Silva, E., J. Schumacher & T. Passler, 2020. Castration of dogs using local anesthesia after sedating with xylazine and subanesthetic doses of ketamine. *Frontiers in Veterinary Science,* **6**, 478.

Soares, J. M., G. F. Avelar & L. R. França, 2009. The seminiferous epithelium cycle and its duration in different breeds of dog (*Canis familiaris*). *Journal of Anatomy,* **215**, 462–471.

Turner, R. M. O., 2007. Pathogenesis, diagnosis, and management of testicular degeneration in stallions. *Clinical Techniques in Equine Practice,* **6**, 278–284.

Yoon, H. Y., J. H. Lee & S. W. Jeong, 2012. Long-term follow-up after implantation of autologous adipose tissue derived mesenchymal stem cells to treat a dog with stifle joint osteoarthrosis. *Journal of Veterinary Clinics,* **29**, 82–86.

Paper received 04.12.2024; accepted for publication 07.04.2025

Correspondence:

Alaa Mohamed B.V.Sc.
Theriogenology Department,
Faculty of Veterinary Medicine,
Cairo University, Egypt 12211,
e-mail: alaa.mohamed@cu.edu.eg;
alamhmd8@gmail.com