



Mesenchymal Stem Cells Enhance Vascular Endothelial Growth Factor-A, Endothelial Nitric Oxide Synthetase, and HSP70 Expression in Improving Erectile Dysfunction in Streptozotocin-induced Diabetic Rats

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Abstract

AIM: This study investigated the therapeutic role of mesenchymal stem cells (MSCs) on erectile function in a diabetes mellitus erectile dysfunction (DMED) rat model by analyzing the expression of endothelial nitric oxide synthetase (eNOS), vascular endothelial growth factor A (VEGF-A), and the 70 kilodalton heat shock proteins (HSP70).

METHODS: MSCs were isolated from umbilical cords (UCs), and their characteristics identified by flow cytometry and osteogenic differentiation analysis. Thirty 8-week-old rats were divided into four groups: Sham, control, T1, and T2. After a 16 h fast, 24 rats were randomly selected and intraperitoneally injected with streptozotocin (STZ) to induce diabetes mellitus. At 8 weeks after STZ injection, rats with DMED were classified into four groups, sham, control group (DMED rats received 500 μ L phosphate buffer saline [PBS]); T1 (DMED rats treated with 500 μ L PBS containing 1×10^6 UC-MSCs); and T2 (DMED rats treated with 500 μ L PBS containing 2×10^6 UC-MSCs). Eight weeks after MSCs administration, the rats' erectile function was measured by cavernous nerve stimulation. The blinded histological and gene expression assessment were used to analyze the eNOS, HSP70 content, and VEGF-A expression on the penile tissues.

RESULTS: MSCs administration, rats in T1 and T2 groups showed a significant enhancement of erectile response that showed a trend of increase of VEGF-A mRNA level expression was 2.2 ± 0.61 in T2 Group supported with the optimum recovery of eNOS, in which the value of eNOS expression was $20.66\% \pm 2.32\%$. While optimum decrease of HSP70 content, the value of HSP70 expression was $15.50\% \pm 0.90\%$. IHC results showed that the DMED induction in rats caused a significant decrease of eNOS content in corpus cavernosum tissue.

CONCLUSION: MSCs could ameliorate DMED in rats by increasing VEGF-A and decreasing HSP70 and eNOS, indicating these cells offer a potential application for DMED patients' treatment.

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Introduction

Erectile dysfunction (ED) is a more prevalent diabetes mellitus (DM) complication, affecting up to 85% of male DM patients [1], [2], [3]. DM-induced erectile dysfunction (DMED) is more severe and commonly appears as ED in nondiabetic patients [4], [5]. The endothelial injury, decrease of smooth muscle content, neuropathy, and fibrosis are responsible

for DMED [6]. Selective phosphodiesterase type-5 inhibitors (PDE5i) constitute the first-line agent for ED; however, it has much lower efficacy in treating patients with DMED [6], [7]. In this condition, revealing the novel alternative approach is a major needed to ameliorate DMED development. Over the past years, stem cell transplantation was considered a potential alternative strategy due to its capability to regulate the inflammatory milieu and recover the functional cells and tissues [8].

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) are ubiquitous fibroblast-like-multipotent stromal cells that attract considerable interest because they can be easily isolated and abundantly assessed from the UC tissues through standardized procedures and have low immunogenicity potential [9], [10]. These cells could be described by high surface antigens levels, such as CD90, CD105, CD73, CD29, and low levels of CD31, CD34, CD45, CD14, CD11b, and HLA-DR [11], [12]. They can differentiate into specific cell lineages, including osteocytes, adipocytes, chondrocytes, and neurons [13]. Other terrific potencies of UC-MSCs are the immunomodulatory and regeneration properties through secreting various trophic factors [14]. The previous studies revealed that MSCs could induce endothelial nitric oxide synthetase (eNOS) [15], which has a beneficial function in maintaining the erectile function, suggesting that MSCs could be a promising strategy to treat DMED.

eNOS is a nitric oxide synthase (NOS) isoform which can produce nitric oxide (NO) continuously through short-term calcium-dependent [15]. Moreover, the expression of eNOS occurs in response to several molecules, including vascular endothelial growth factor A (VEGF-A) [16]. A recent study revealed that the DMED pathogenesis is due to an interfered NO secretion, in which eNOS has a major role in inhibiting the profibrotic consequences of hyperglycemia in penile tissues [6]. Another study revealed several vascular injuries, such as vascular smooth muscle and endothelial cell damage in the corpus cavernosum of DMED patients [8]. The sufficient VEGF-A is the main factor in improving vascular repair [17]. This condition could improve cellular repair characterized by decreased levels of cellular stress, characterized by the expression of The 70 kD heat shock proteins (HSP70) [18]. Moreover, other studies have revealed the capability of MSCs in releasing VEGF-A to restore vascular tissues injury [16], [19], [20]. However, more impressive explanations regarding the high expression of VEGF-A and the mechanisms of MSCs in upregulating VEGF-A expression in injured tissues remain unclear.

This study builds a condition of streptozotocin (STZ)-induced DMED in the rat model. We evaluated the underlying mechanism of MSCs in improving the erectile function of DMED rats by analyzing the expression of eNOS, VEGF-A, and HSP-70.

Materials and Methods

Animals and ethical statement

Animal models complied with regulations of the Ethical Committee of the Faculty of Medicine, Universitas Sumatera Utara. The Ethical Committee approved this

study (number 370/KEP/USU/2020). A total of thirty male Sprague–Dawley rats (8 weeks- old) weighting 200–250 g were purchased from Lembaga Penelitian dan Pengujian Terpadu, Unit IV, Universitas Gadjah Mada. After a 16 h fast, 24 rats were randomly selected and intraperitoneally injected with (STZ, 60 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). The remaining six rats were used as sham (rats with no DMED received 500 μ L phosphate buffer saline [PBS]).

Assessment of rats' blood glucose and body weight

The blood glucose levels and body weights of rats were analyzed at the beginning and end of treatment. The blood glucose level was measured by a pharmaceutical-grade glucometer (Accu-Check; Roche, Basel, Switzerland). For DM validation, the blood glucose was measured on the 3rd day after STZ induction. Of the 24 rats, 21 (87.50%) were diabetic with higher than 300 mg/dl fasting glucose levels.

Assessment of ED

At 8 weeks after STZ injection, apomorphine (APO, 100 μ g/kg; Sigma-Aldrich, St. Louis, MO, USA) was used to select the ED of rats according to Heaton's method [21]. Rats with DMED were present in 18 of the 21 (85.71%) and identified by unresponsive erectile stimulation within 30 min.

Administration of MSC's

Four weeks after DMED were induced, 24 rats were randomly assigned into four groups (six rats in each group): Sham (placebo or no treatment group); control (DMED rats received 500 μ L PBS); T1 (DMED rats treated with 500 μ L PBS containing 1×10^6 UC-MSCs); and T2 (DMED rats treated with 500 μ L PBS containing 2×10^6 UC-MSCs). MSC intervention with a 25G needle was carried out at week 8 (after validation) with a saline volume of 300 μ L. All rats received low-dose anesthesia before receiving the intracavernous injection. MSCs were injected 1 time for 4 weeks intracavernous which was divided right and left in the corpus cavernosum to spread throughout the tissue. Then the rats were reared until week 12. ED validation was also carried out before the rats were sacrificed to analyze the changes before and after treatment.

UC-MSCs isolation and culture

UCs were collected from 19-day pregnant female rats, and the MSCs were isolated and cultured according to previous standardized methods [10]. In brief, UC tissue was cut into smaller pieces under aseptic conditions. A UC tissue section was planted

into a culture flask containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Louis St, MO), supported with 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco™ Invitrogen, NY, USA), 0.25% amphotericin B (Gibco™ Invitrogen, NY, USA), 10% fetal bovine serum (FBS) (Gibco™ Invitrogen, NY, USA), and cultured at 37°C in a humidified atmosphere consisting 5% CO₂. The culture medium was replaced every 3 days. After reaching approximately 80% confluence, UC-MSCs-like in culture flasks was passage into a new culture flask. UC-MSCs-like at passage 4 was used for further studies.

Flow cytometry

UC-MSCs-like membrane antigens at passage 4 were characterized using flow cytometry. Briefly, after UC-MSCs were passaged and washed, the cells were stained for 30 min using PBS containing anti-CD90.1-PerCP (cat. # 557266; BD, San Diego, CA, USA), anti-CD29-alexa fluor 647 (cat. # 562153; BD, San Diego, CA, USA), anti-CD45-FITC (cat. # 554877; BD, San Diego, CA, USA), and anti-CD31-PE (cat. # 555027; BD, San Diego, CA, USA) in the dark at room temperature. The cells were then rinsed twice using PBS. The flow cytometry and post-acquisition analysis were performed and calculated by a BD Accuri C6 Plus flow cytometer (BD, San Diego, CA, USA) and BD Accuri C6 Plus software (BD, San Diego, CA, USA).

Differentiation of UC-MSCs

We further performed the osteogenic differentiation of UC-MSCs-like in passage 4. The UC-MSCs-like were expanded in standard medium containing DMEM that augmented with 10% FBS, 1% penicillin/streptomycin (100 U/mL; respectively) and 0.25% amphotericin B (62.5 µg/mL) at 37°C, 5% CO₂ and humidified atmosphere until 95% confluency. An osteogenic differentiation medium made of MesenCult™ osteogenic basal medium (Rat) (cat. # 05505; Stem Cell Technologies, Singapore) augmented with 20% MesenCult™ Osteogenic Stimulatory Supplement (Rat) (cat # 05506; Stem Cell Technologies), 1% L-Glutamine (Gibco™ Invitrogen), 1% penicillin/streptomycin (100 U/mL; respectively) (Gibco™ Invitrogen), and 0.25% amphotericin B (62.5 µg/mL) (Gibco™ Invitrogen), was a standard medium substitute. This osteogenic medium was replaced every 3 days. Alizarin red staining was performed followed by 21 days of osteogenic induction of MSCs (Sigma-Aldrich, Louis St, MO). The deposition of calcium was appeared in red color.

On the other side, for inducing adipogenic differentiation, the UC-MSCs-like with 95% confluency was cultured using adipogenic differentiation medium which made of MesenCult™ MSC Basal Medium (Rat) (cat # 05505; Stem Cell Technologies), MesenCult™

Adipogenic Differentiation Supplement (Rat) (cat # 05509; Stem Cell Technologies), 1% L-Glutamine (Gibco™ Invitrogen), 1% penicillin/streptomycin (100 U/mL; respectively) (Gibco™ Invitrogen), and 0.25% amphotericin B (62.5 µg/mL) (Gibco™ Invitrogen). The medium was changed every 3 days. After 14 days of adipogenic induction, the oil red staining was poured into cultured plates. The lipid deposition was shown in red color.

Immunostaining analysis

Six rats from each group receiving 80 mg/kg ketamine and 5 mg/kg xylazine were sacrificed after treatments. Half of the penile tissues were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C. Penile tissue slices were deparaffinized in xylene and rehydrated using sequential ethanol (100%, 95%, 80%, 70%, and 50%). Peroxidase blocking activity was employed by 3% H₂O₂ incubation. The tissues were incubated by following antibodies, such as anti-rabbit-eNOS polyclonal antibody (dilution 1:100, Cat. FNab10162, Fine Test, Wuhan, China) and anti-rabbit-HSP-70 polyclonal antibody (dilution 1:100, Cat. A12948, ABclonal, Wuhan, China) overnight at 4°C. Secondary antibody staining was performed using biotinylated anti-rabbit IgG (Ref. STU700L10, Biocare Medical, CA, USA) at room temperature for 2 h. The semiquantitative data analysis was employed using ImagePro Plus software (Media cybernetics, Inc.).

VEGF-A relative expression analysis

After thawing the frozen 100 mg penile tissues, RNA was later extracted. TRI-Reagent (Sigma-Aldrich, Dorset, UK) was carried out to extract total RNA according to the instructions given, as used in our previous study [21]. The RNA sample was incubated by DNase I (Sigma-Aldrich, Dorset, UK) to abolish DNA contamination. Reverse transcription was performed with the Enhanced Avian First Strand cDNA Synthesis Kit (Sigma-Aldrich, Dorset, UK) per the manufacturer's instructions. The oligo d[T] primer was used for reverse transcription, and the sample reaction mix was incubated at 70°C for 10 min and at 45°C for 30 min. The polymerase chain reaction (PCR) for cDNA synthesis was employed using eco real-time PCR system (Illumina Inc., San Diego CA, USA).

The quantitative PCR analysis of VEGF-A and beta-actin as housekeeping gene was performed by KAPA SYBR® FAST Universal Kit (Sigma-Aldrich, Dorset, UK) using each oligonucleotide primer (VEGF-A forward 5'-CTGCTGTGTTGGGTGCACTGG-3' and VEGF-A reverse 5'-GGTTTGATCCGCATAATGTGCAT-3'; beta-actin forward 5'-ATTGGCAATGAGCGGTTCCGC-3' and reverse 5'-CTCCTGCTTGCTGATCCACATC-3'). All samples and reagents were mixed and incubated

using Eco Real-Time PCR System at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The mRNA expression analysis was performed using Eco Study Software (Illumina Inc., San Diego CA, USA).

Statistical analysis

All data are displayed as means ± standard deviation. The data calculation was employed using SPSS 23.0 (IBM Corp., NY, USA). The difference statistical significances between groups were calculated using one-way ANOVA, followed by Bonferroni *post hoc* analysis. $p < 0.05$ was considered significant.

Results

Measurement of rats' blood glucose and body weight

The final weight of STZ-induced diabetic rats on the control groups was markedly lower than normal rats. The blood glucose level of all diabetic rats was totally higher compared to sham. After MSCs administration, the final weight of the T1 and T2 was significantly higher than the control ($p = 0.0413$ for T1 and $p = 0.0309$ for T2). On the other hand, the final blood glucose level of the T1 and T2 groups was also

significantly lower than control ($p = 0.0192$ for T1 and $p = 0.0079$ for T2; Table 1).

Table 1: Bodyweight and blood glucose variable

Variable	Sham	Control	T1	T2
Initial weight (g)	213.6 ± 13.8	208.8 ± 12.2	209.6 ± 13.0	211.8 ± 10.3
Final weight (g)	346.6 ± 29.3*	194.2 ± 8.2	231.6 ± 22.4*	293.8 ± 7.3*
Initial glucose (mg/dL)	170.2 ± 11.9	156.8 ± 10.5	164.7 ± 12.4	174.7 ± 8.6
Final glucose (mg/dL)	140.2 ± 25.9*	512.8 ± 47.2	431.4 ± 27.0*	265.4 ± 64.0*

Values are expressed as mean ± SD * $p < 0.05$ compared to the control group. SD: Standard deviation. n = 6/group.

Characteristics and differentiation capability of MSCs

MSCs from the UC were analyzed for plastic-adherent potential under standard culture conditions, antigen-specific surface markers, and differentiation capability after four passages. In this study, the MSCs morphology at the fourth passage exhibited specific monolayers of spindle fibroblast-like and adherent capacity to the plastic culture (Figure 1a). The osteogenic and adipogenic differentiation capability of MSCs was analyzed by culturing the MSCs under osteogenic and adipogenic differentiation medium for 21 and 30 days, respectively. After incubation, the calcium and adipose deposition were visualized as a red color after alizarin red and oil red o solution administration, respectively (Figure 1b and c). On the other hand, to characterize MSCs surface antigens, we performed flow cytometry analysis regarding the International Society for Cellular Therapy. We found a high level of CD90.1 (99.3 ± 0.54%) and CD29 (95.2 ± 0.73%) and negative

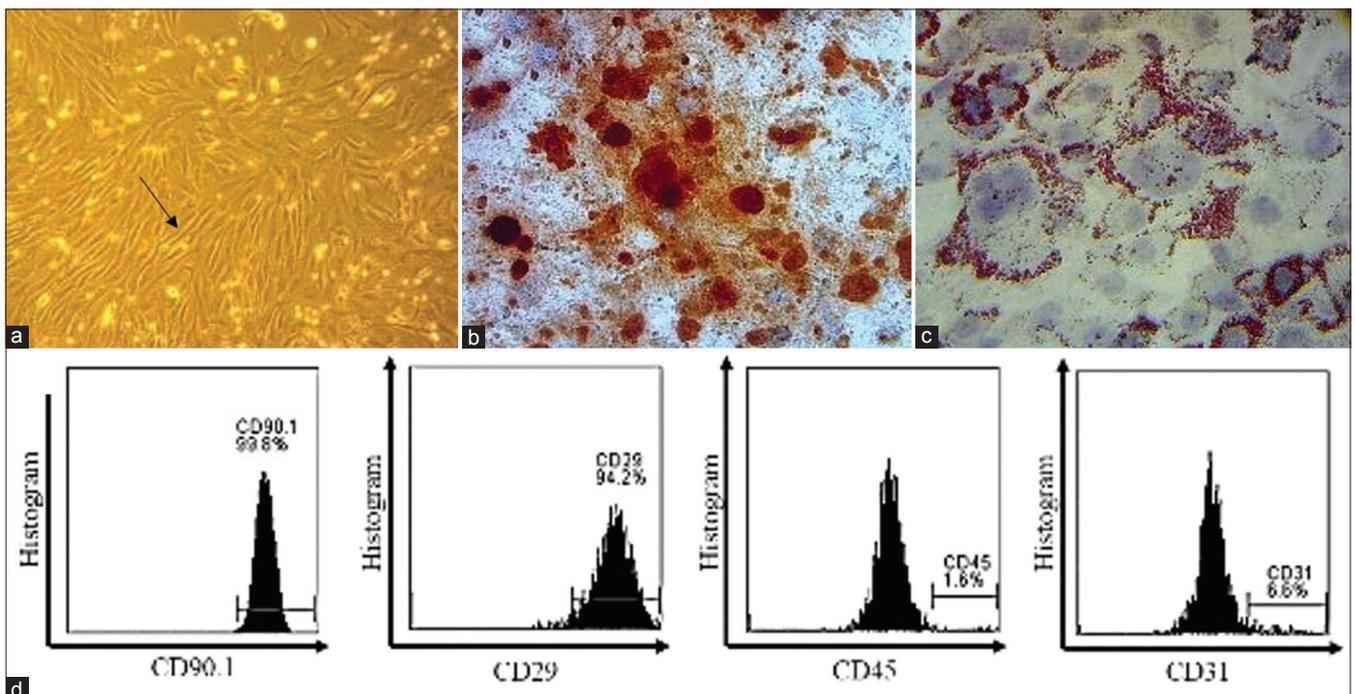


Figure 1: Mesenchymal stem cell (MSCs) candidates showed spindle forms such as fibroblast-like cells (100× magnification) (a). MSCs were incubated by osteogenic and adipogenic differentiation medium to evaluate the capability of MSCs to differentiate into osteocyte and adipocyte, respectively. The calcium deposits were positively stained and represented in red color after alizarin red staining (b), and the adipose deposition was also represented in red color after oil red staining (c). MSCs also expressed CD90.1, CD29, and lacked the expression of CD31 and CD45 after flow cytometry analysis (d)

expression of CD31 ($5.62 \pm 1.27\%$) and CD45 ($1.66 \pm 0.24\%$; Figure 1d).

MSCs enhance the erectile function of DMED rat models

Erectile function measurement showed that DMED induction decreased erectile response significantly in rats. After administration of MSCs, the erectile response experienced a significant increase compared to the control group (0.33 ± 0.52), where the erectile response in the T1 group was 19.67 ± 1.63 ($p = 0.0021$) and the T2 group was 25 ± 2.45 ($p = 0.0014$) within 30 min. Whereas, the erection response of the sham group was 6.83 ± 0.75 ($p = 0.0093$). In which the fastest recovery was on the T2 with a value of (Figure 2).

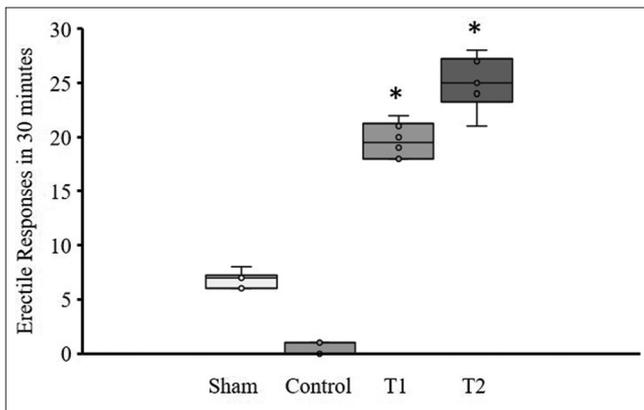


Figure 2: Erectile function measurement in rats with diabetes mellitus erectile dysfunction (DMED) in each group ($n = 6$ /group). Graph summarizing the quantitative data of erectile responses DMED rats in 30 min and expressed as mean \pm standard deviation. * $p < 0.05$ compared with control group

MSCs administration increases eNOS expressions in corpus cavernosum tissue

IHC results showed that the DMED induction in rats caused a significant decrease in the expression of eNOS in corpus cavernosum tissue ($p = 0.0273$). After

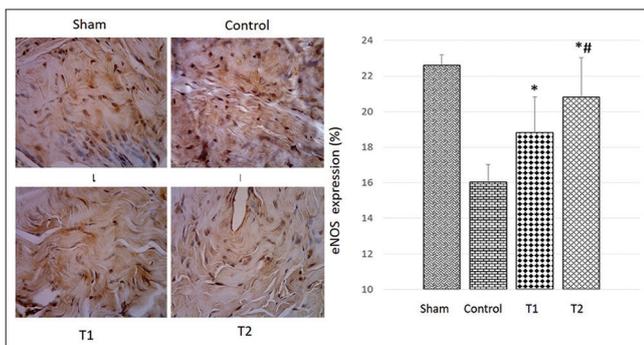


Figure 3: eNOS expression in corpus cavernosum tissue from each group ($n = 6$ /group). Representative images of eNOS expression in the corpus cavernosum tissue original magnification: $\times 400$. Graph summarizing the quantitative data of eNOS content in corpus cavernosum tissue and expressed as mean \pm standard deviation. * $p < 0.05$ compared with control

MSCs administration, rats on T1 showed a partial recovery of eNOS expression ($p = 0.0467$). The T2 group showed an optimum recovery of eNOS, in which the value of eNOS expression was $20.66 \pm 2.32\%$ (Figure 3a and b).

MSCs administration decrease HSP70 expression in corpus cavernosum tissue

IHC results showed that the DMED induction in rats caused a significant increase in the expression of HSP70 in corpus cavernosum tissue ($p = 0.0402$). After MSCs administration, rats on T1 showed a significant decrease of HSP70 expression ($p = 0.0110$). Moreover, the T2 showed an optimum decrease of HSP70 content, in which the value of HSP70 expression was $15.50 \pm 0.90\%$ (Figure 4a and b).

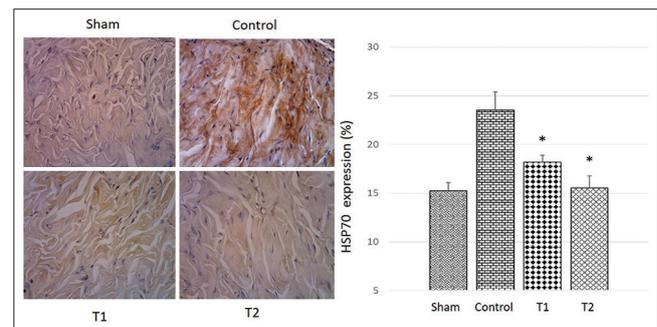


Figure 4: HSP-70 expression in corpus cavernosum tissue from each group ($n = 6$ /group). Representative images of HSP-70 expression in the corpus cavernosum tissue original magnification: $\times 400$. Graph summarizing the quantitative data of HSP-70 content in corpus cavernosum tissue and expressed as mean \pm standard deviation. * $p < 0.05$ compared with control

MSCs administration increases VEGF-A mRNA level in corpus cavernosum tissue

qRT-PCR results showed that the DMED induction in rats caused a significant decrease of VEGF-A mRNA content in corpus cavernosum tissue ($p = 0.0364$). After MSCs administration, rats on the T1 showed a trend of increase of VEGF-A mRNA level expression. Moreover, the T2 showed an optimum significant increase of VEGF-A mRNA level content, in which the value of VEGF-A mRNA level was 2.2 ± 0.61 ($p = 0.0092$; Figure 5).

Discussion

DMED is a complex vascular disease characterized by prolonged hyperglycemia on the corpus cavernosum, nerves, and blood vessels, and the dysfunction of the endothelium, leading to penile fibrosis. These severe and complex mechanisms can damage the corpus cavernosum structure, leading to decreased erectile function [22]. A previous study reported that VEGF-A expression and signaling

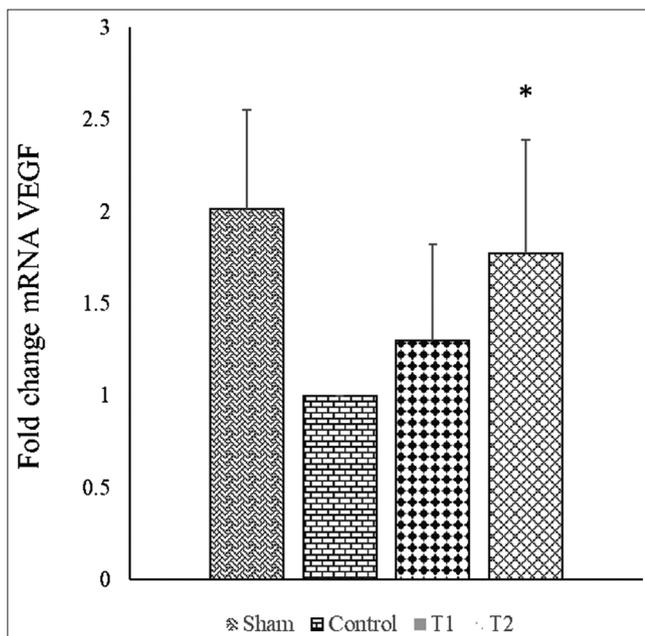


Figure 5: mRNA level of VEGF-A in corpus cavernosum tissue from each group ($n = 6/\text{group}$). Graph summarizing the quantitative data of VEGF-A mRNA level in corpus cavernosum tissue and expressed as mean \pm standard deviation. * $p < 0.05$ compared with control

molecules such as eNOS and NO were decreases in the penile tissue of STZ-induced diabetic rats [2]. On another side, a previous study revealed that chronic hyperglycemia and DM lead to decreased production and activity of NO [16]. NO is established through the L-arginine alteration by NOS, which results in three isoforms: iNOS, nNOS, and eNOS; respectively [16]. In the DMED condition, eNOS was expressed in the smooth muscle cells and cavernosal endothelium, which appeared to be fundamental for erectile function maintenance [23].

In this study, the MSCs treatment of all treatment groups increased the expression of eNOS in DMED rats, in which the T2 group has an optimum enhancement. These results also supported another finding, which reported that the MSCs in all treatment groups could enhance the mRNA VEGF-A expression in penile tissues. The T2 group has optimum expression. These findings suggest that MSCs treatment was effective in promoting the revascularization of corpus cavernosum on DMED rats. A previous study revealed that p-AKT could stimulate endothelial cells to express eNOS, crucial for vasodilatation [15]. The significant enhancement of the level of eNOS in the corpus cavernosum on all treatment groups than control suggested that MSCs could enhance the expression of VEGF-A in the corpus cavernosum to trigger the PI3K-AKT signaling pathway, resulting in the enhancement of eNOS expression. The increase of eNOS could inhibit vasospasm and stimulate the vasodilatation process, thereby restore the blood supply and improve DMED [15].

The previous studies revealed that the improvement of cellular repair could be characterized

by cellular stress levels, such as HSP70 [18]. On the other hand, other studies revealed that endothelial cells avenge with an anti-inflammatory response by producing antioxidant and cytoprotective protein, resulting in the expression of HSP70 [23]. Moreover, another study also reported that HSP70 is essential for angiogenesis through activation of p-Akt signaling in which the inhibition of HSP70 level could decrease the activation of p-Akt [15]. Interestingly, our findings showed that MSCs in all treatment groups decreased the expression of HSP70 in the corpus cavernosum, besides the control group showed an increased level of HSP70. MSCs attenuate the inflammatory condition by expressing several anti-inflammatory molecules [9] and decrease the expression of HSP70 in treatment groups into normal conditions. These conditions could lead to the expression of VEGF-A that promotes vascularization in the corpus cavernosum.

Our experiment does have some places for improvement. In this study, we just performed an animal experiment. We did not analyze the expression of the p-Akt pathway and its blockade mechanism in vitro. We also did not analyze the number of blood vessels in the corpus cavernosum.

Conclusion

MSCs could increase the level of eNOS and VEGF-A levels in DMED induction rats. Therefore, MSCs also reduced the HSP70 level associated with enhancing the erectile function of DMED rat models. This MSCs capacity could become an attractive and great therapy to improve erectile function in diabetic patients.

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