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Original Article

Amelioration of exosome and mesenchymal stem cells in rats infected with diabetic nephropathy by attenuating early markers and aquaporin-1 expression

Melhoria das células-tronco exossomais e mesenquimais em ratos infectados com nefropatia diabética atenuando marcadores precoces e expressão de aquaporina-1

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Abstract

Diabetic nephropathy (DN) is a prevalent diabetic microvascular condition. It is the leading cause of kidney disease in the advanced stages. There is no currently effective treatment available. This research aimed to investigate the curative potentials of exosomes isolated from mesenchymal stem cells affecting DN. This study was performed on 70 male adult albino rats. Adult rats were randomized into seven groups: Group I: Negative control group, Group II: DN group, Group III: Balanites treated group, Group IV: MSCs treated group, Group V: Exosome treated group, Group VI: Balanites + MSCs treated group and Group VII: Balanites + exosome treated group. Following the trial period, blood and renal tissues were subjected to biochemical, gene expression analyses, and histopathological examinations. Results showed that MDA was substantially increased, whereas TAC was significantly decreased in the kidney in the DN group compared to normal health rats. Undesired elevated values of MDA levels and a decrease in TAC were substantially ameliorated in groups co-administered Balanites aegyptiacae with MSCs or exosomes compared to the DN group. A substantial elevation in TNF-α and substantially diminished concentration of IGF-1 were noticed in DN rats compared to normal health rats. Compared to the DN group, the co-administration of Balanites aegyptiacae with MSCs or exosomes substantially improved the undesirable elevated values of TNF-α and IGF-1. Furthermore, in the DN group, the mRNA expression of Vanin-1, Nephrin, and collagen IV was significantly higher than in normal healthy rats. Compared with DN rats, Vanin-1, Nephrin, and collagen IV Upregulation were substantially reduced in groups co-administered Balanites aegyptiacae with MSCs or exosomes. In DN rats, AQP1 expression was significantly lower than in normal healthy rats. Furthermore, the groups co-administered Balanites aegyptiacae with MSCs or exosomes demonstrated a substantial increase in AQP1 mRNA expression compared to DN rats.

Keywords: diabetic nephropathy, exosome, AQP1, Vanin1, nephrin, collagen IV.

Resumo

A nefropatia diabética (ND) é uma condição microvascular diabética prevalente. É a principal causa de doença renal em estágios avançados. Atualmente, não há tratamento eficaz disponível. Esta pesquisa teve como objetivo investigar os potenciais curativos de exossomos isolados de células-tronco mesenquimais que afetam a ND. Este estudo foi realizado em 70 ratos albinos adultos machos. Ratos adultos foram randomizados em sete grupos: Grupo I: Grupo de controle negativo, Grupo II: Grupo DN, Grupo III: Grupo tratado com Balanites, Grupo IV: Grupo tratado com MSCs, Grupo V: Grupo tratado com exossomos, Grupo VI: Grupo tratado com Balanites + MSCs e Grupo VII: Balanites + grupo tratado com exossomas. Após o período experimental, o sangue e os tecidos renais foram submetidos a análises bioquímicas, de expressão gênica e exames histopatológicos. Os resultados mostraram que o MDA aumentou substancialmente, enquanto o TAC diminuiu significativamente no rim no grupo DN em comparação com ratos saudáveis normais. Valores elevados indesejados de níveis de MDA e uma diminuição no TAC foram substancialmente melhorados em grupos coadministrados *Balanites aegyptiacae* com MSCs ou exossomas em comparação com o grupo DN. Uma elevação substancial em TNF-α e uma concentração substancialmente diminuída de IGF-1 foram observadas em ratos DN em comparação com ratos saudáveis normais. Em comparação com o grupo DN, a coadministração de *Balanites aegyptiacae* com MSCs ou exossomas melhorou substancialmente os valores elevados indesejáveis de TNF-α e IGF-1. Além disso, no grupo DN a expressão de mRNA de vanina-1, nefrina e colágeno IV foi significativamente maior do que em ratos saudáveis normais. Comparado com ratos DN, Vanin-1, Nephrin e colágeno IV Upregulation foram substancialmente reduzidos em grupos co-administrados *Balanites aegyptiacae* com MSCs ou exossomos. Em ratos DN, a expressão de AQP1 foi significativamente menor do que em ratos saudáveis normais. Além disso, os grupos que coadministraram *Balanites aegyptiacae* com MSCs ou exossomos demonstraram um aumento substancial na expressão de mRNA de AQP1 em comparação com ratos DN.

Palavras-chave: nefropatia diabética, exossoma, AQP1, Vanin1, nefrina, colágeno IV.

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1. Introduction

Diabetic nephropathy (DN) is considered a microvascular condition associated with poor control of blood glucose in subjects with types I and II diabetes. It eventually develops into an end-stage kidney disorder, affecting approximately 40% of patients requiring replacement treatment for the kidneys (Alicic et al., 2017). For the treatment of DN, there is no definite approach or target. Hyperglycemia-induced glomerular podocyte injury is hypothesized to be a critical early stage in DN. In addition, high hyperglycemia promotes albuminuria by lowering podocyte counts and inducing apoptosis of cultured podocytes (Li et al., 2013).

Hemodynamic alterations, mesangial cell growth, oxidative stress, generation of fibrosis, and glomerulosclerosis are among the structural and functional modifications implicated in the aetiology of DN (Forbes et al., 2008). Despite traditional therapies that improve high blood glucose levels and hypertension, potential curative approaches can avoid and reverse DN development (Lorenzen et al., 2011; Fernandez-Fernandez et al., 2014; Ahmad, 2015). Diabetic complication progression occurs due to the production of reactive oxygen species (ROS), mostly superoxide radical ($O_2^{\bullet-}$), which enhances cell dysfunction and oxidative damage through protein denaturation, lipid peroxidation, and DNA mitochondrial damage (Forbes et al., 2008). Changes in ATP generation, intracellular calcium imbalance, and cell membrane permeability in cells of the kidneys involving epithelial renal cells, mesangial cells, and endothelial glomerular cells contribute to apoptosis or necrosis, resulting in cell death (Vallon and Thomson, 2012).

The study of medicinal plants is necessary to enhance effective herbal medicine utilization and evaluate their effectiveness as a means of novel medications (Parekh and Chanda, 2007). Balanites aegyptiacae is utilized in many folk medicines in India and Asia. Balanites aegyptiacae mesocarp fruit extract can also be utilized as a supplemental natural antidiabetic substance to help prevent diabetes complications. This approach appears promising in terms of preventing or delaying DN. Balanites mesocarp fruit extract significantly affects many target tissues involving kidneys and has a positive effect on mediators of severe vessel injury (EL-Shater et al., 2018).

An alternative approach to diabetes treatment is cell-based therapy. Exosomes isolated from mesenchymal stem cells have already been identified as a distinct cell-to-cell communication mechanism that has evolved into a critical paracrine mediator (Bruno et al., 2009). Extracellular vesicles (EVs), known as exosomes, are produced by cells as part of the endocytic cascade and released into the extracellular environment (Skottvoll et al., 2018). Exosomes produced from an endosome membrane invagination in order to generate multivesicular bodies, or intraluminal vesicles, within the endosome are then released when such endosomes and the plasma membrane bind together (Lener et al., 2015). Exosomes which include lipids, metabolites, and proteins from plasma membranes and cytosol of the cell, along with nucleic acids (e.g., DNA, mRNA, and noncoding RNA), are known to be involved in cell-cell communication, protein/nucleic acid transfer, coagulation, and antigen presentation (Li et al., 2017).

Pluripotent mesenchymal stem cells can regenerate, proliferate, self-renew, and differentiate into several types of cells (Charbord, 2010). MSCs are distinguished by their ability to differentiate into osteocytes, chondrocytes, and adipocytes and their MSC marker expression (Kwon et al., 2016).

The research aimed to assess the potential roles of exosomes isolated from MSCs in DN pathogenesis and histological changes, as well as to investigate the improvement of abnormal renal function associated with DN by aqueous *Balanites aegyptiacae* fruit extract, with a focus on its prospective regenerative properties on β-cells.

2. Material and Methods

2.1. Plant

The *Balanites aegyptiacae* fruits were obtained from Haraz for herbs (Egypt, Cairo) and were identified at the Department of Botany Taxonomically, Faculty of Science at Zagazig University, Egypt. Dates are dark brown in colour, and the fleshy pulps of unripe and ripe fruits can be consumed as dried or fresh. The seeds were discarded after the fruit flesh was sliced and weighed.

2.2. Chemicals

The Company of Sigma Aldrich Chemicals (St. Louis, Mo., USA) provided streptozotocin (STZ) as well as nicotinamide (NIC), which were kept at -20°C away from light.

2.3. Preparation of MSCs derived exosomes

Exosomes extracted from MSCs were isolated using the supernatants of MSCs - conditioned media. First, MSCs extracted from rat bone marrow were prepared in the Medical Experimental Research Center (MERC) in the Faculty of Medicine, Mansoura University, Egypt. MSCs were cultured overnight in Dulbecco's Modified Eagle Medium (DMEM) without fetal bovine serum and with 0.5% human serum albumin (Sigma-Aldrich), MSCs were cultivated overnight. Trypan blue exclusion revealed that the cell-cultured overnight would have more than 99% viability. Cell plating was done for seven days at a density of 4000 cells per cm^2. On day seven, cells were trypsinized using 0.25% trypsin in one mmol per litre EDTA at 37°C for 5 min, counted, and then replated in growth media at 2000 cells per cm^2 for an additional period of seven days (end of passage 1) (Bruno et al., 2012). The conditioned media were collected and stored at -80°C.

2.4. Isolation of MSC-derived exosomes

MSCs were cultured in a normal growth medium until reaching 80% confluence, and a serum-free medium was substituted for the medium. After 48 hours, the cell culture media was collected and centrifuged at 300 xg for 10 min at 4°C, followed by 2000 xg for 10 minutes, then 10000 xg for 30 minutes to remove any remaining cells. Twenty ml supernatant was centrifuged at 100,000 xg (Sorvall SureSpin 630) for 70 minutes at 4°C to isolate exosomes.

This resulting pellet was rinsed using an equal volume of ice-cold PBS before being centrifuged for 70 minutes at 100,000 xg 4°C, which contained the EVs. The exosome-containing pellet was then resuspended in (50 to 100 µl) of PBS (Théry et al., 2006).

2.5. Preparation of aqueous extract of Balanites aegyptiacae fruits

The coat epicarp of collected *Balanites aegyptiacae* fruits was carefully removed manually, and also the fruit's mesocarp was subsequently peeled with a cleaned, dried knife. The fleshy outer layer named mesocarp then detached from the seeds. The dried mesocarp in the air was then ground with a coffee grinder in the laboratory. Afterward, it was kept in the refrigerator in a dry plastic container even after it was needed. Following the removal of the seeds, 4 kg of dried fruit was immersed in distilled H_2O for 24 hours (200 ml distilled H_2O was used to extract 100-gram powder). This freshly made filtrate passed freeze-drying (utilizing labconco, model 18, freeze dryer) to yield a thick dark brown extract. Prepared aqueous extract of *Balanites aegyptiacae* fruit was administered orally by an oral gastric tube every day for four weeks at a dose of 80 mg/kg body weight (El Deib and Ali, 2018).

2.6. Laboratory animals

Seventy male adult albino rats weighing 180 – 200 g were housed in the Experimental Animal House of the Faculty of Science, Zagazig University. Rats were placed in a controlled setting with a 25 °C temperature, a 65% relative humidity, or a 12-hour cycle of light and dark. The rats provided unlimited obtaining commercial pellet rat chaw as well as tap water. The Zagazig University's Ethical Committee approved the study design as well as procedures (ZU-IACUC/1/F/80/2019).

2.7. Induction of DN in adult male albino rats

Diabetic nephropathy (DN) was intraperitoneally induced utilizing a single freshly prepared Streptozotocin injection (60 mg/kg b. wt, STZ) dissolved in (100mM, pH 4.5) cold citrate buffer in overnight-fasted adult rats following 15 minutes of intraperitoneal (i.p) NIC injection (120 mg/kg body weight, NIC) dissolved in 0.9% (wt /v) sodium chloride (Punitha et al., 2005). After one hour of streptozotocin and NIC injection, rats received an overnight 5% glucose solution to prevent hypoglycemia. Afterward, the levels of fasting blood glucose were monitored with a portable glucose metre for 72 hours following injection as well as on the seventh day. Diabetic rats with fasting blood sugar levels higher than 250 mg/dl (Deeds et al., 2011). Compared with controls, male adult albino rats demonstrated substantially increased serum creatinine and urea levels, as well as histological alterations, confirmed nephropathy at the end of the sixth week after induction. This study involved rats with these significant values (Ebrahim et al., 2018).

2.8. Experimental design

Seventy male adult albino rats were randomly categorized into seven groups, with ten animals each.

Group I: Negative control group: included normal health rats.

Group II: Positive control group: Rats with DN.

Group III: Balanites treated group: DN was induced, and rats were treated with the prepared *Balanites aegyptiacae* fruit aqueous extract administered orally by an oral gastric tube at a dose of 80 mg/kg b. wt daily for four weeks (El Deib and Ali, 2018).

Group IV: MSCS treated group: At four weeks following STZ injection, DN rats were treated with 1.0×10^4 MSCs per gram b. wt per animal is suspended in 200 µ L phosphate buffer saline (PBS) twice every four weeks via the tail vein (Nagaishi et al., 2016).

Group V: Exosome-treated group: DN was induced in rats, and they were given twice exosome injections (100 µg per kg per dose dissolved in 200 µ L phosphate buffer saline (PBS)) intravenously (Yang et al., 2015). The first was administered during the eighth week of the study, and the second was administered at the end of the tenth week (Ebrahim et al., 2018).

Group VI: Balanites + MSCs treated group: DN was induced in rats, and they received prepared *Balanites aegyptiacae* fruit aqueous extract of (80 mg/kg) orally for four weeks daily (El Deib and Ali, 2018) and MSCs (1.0×10^4 cell/g) two times every four weeks via the tail vein (Nagaishi et al., 2016).

Group VII: Balanites + exosomes treated group: DN received prepared aqueous extract of *B. aegyptiacae* fruit (80 mg/kg) orally for four weeks daily (El Deib and Ali, 2018) and twice exosome injection (100 µg per kg per dose dissolved in 200 µ L ml PBS) (Yang et al., 2015). The first was administered during the eighth week of the study, and the second was administered at the end of the tenth week (Ebrahim et al., 2018).

2.9. Specimen collection

Rats fasted for 10 hours after the trial ended and then euthanized using urethane. To evaluate TNF- α and IGF-1, blood samples per group were collected in a free anti-coagulant tube from the retro-orbital venous plexus and then centrifuged at 4000 rpm for 20 minutes. All specimens were kept at -20 °C until biochemical analysis. Each rat's kidney tissue was removed, washed using saline solution, blood removed, and sliced into three portions. The initial portion of kidney tissue per rat was kindly homogenized in 2mL of PBS (pH 7.4) utilizing a Teflon homogenizer and then centrifuged for 15 minutes at 4°C at 3500 rpm. In the supernatants, the TAC and MDA concentrations were measured. The following kidney tissue fragment was submerged in 10% neutral buffered formalin for histopathological analysis. In contrast, the final kidney tissue section was stored at -80 °C until genetic material RNA was isolated.

2.10. Biochemical parameters

2.10.1. Determination of antioxidant biomarkers as well as oxidative stress in tissue homogenate

The homogenized kidney tissue sample was utilized to determine the antioxidant biomarkers as well as oxidative stress. The amount of lipid peroxidation was

monitored by colorimetrically measuring MDA levels with a commercially available kit from Biodiagnostic Company, Egypt (Ohkawa et al., 1979). The TAC was quantified by a commercially available kit from Biodiagnostic Company, Egypt (Koracevic et al., 2001).

2.10.2. Determination of serum TNF-α and insulin-like growth factor -1

Serum level of TNF-α was measured (Brouckaert et al., 1993) utilizing an available commercial ELISA kit (RayBio® Rat, Norcross, GA, USA: RayBiotech) Catalog #: ELR-TNFα in accordance with manufacturer guidelines. In addition, RAT/MOUSE IGF-I Quantikine® ELISA was used to assess (Nitert et al., 2005) the serum IGF-I concentration.

2.10.3. Histopathological examination for kidney

After sacrification, kidney samples were instantly placed for 24 hours in 10% neutral buffered formalin. The fixed, processed samples were cut into 4-m-thick paraffin sections. In order to investigate kidney histology, the sliced sections were stained with hematoxylin and eosin (H&E). The slides were then examined under a light microscope (Palipoch and Punsawad, 2013).

2.10.4. Morphometry

A semi-quantitative analysis of sections was performed in order for histopathological assessment of the severity of kidney lesions (Houghton et al., 1978): Score 0 indicated normal, score + indicated a level between normal and mild, score ++: mild level; <25% of the total fields analyzed showed histopathological changes, Score +++: moderate level; <50% of the total fields analyzed showed changes, and score ++++: severe level; < 75% of the total fields analyzed showed histopathological changes. The scoring system is based on histological injury in the glomerular, endothelial, tubular, and interstitial components.

2.10.5. Quantitative real-time polymerase chain reaction (RT-qPCR)

Following the manufacturer's instructions, isolation of total RNA from kidney tissue samples was performed using TRIzol™ Reagent (USA: Invitrogen). In order to determine the purity and concentration of isolated genetic material RNA, absorbance at 260/280 nm was measured using a Beckman dual spectrophotometer (USA). RNA samples with an A260/A280 ratio of 1.9 to 2.1 were used for further investigation. Reverse transcription of isolated RNA into complementary DNA was done utilizing an elevated-capacity cDNA synthesis kit from Qiagen in the United States. Quantitative real-time PCR was carried out to assess the relative mRNA expression of nephrin, vanin-1, collagen IV, and aquaporin-1 (AQP1) using real-time PCR equipment (Biometra, Germany) and an SYBR Green Master Mix kit (Qiagen, Germany). The following instructions were used to programme the real-time PCR thermal cycler: The annealing temperature for nephrin and vanin-1 sets was maintained at 60 °C. They were subjected to the following thermal characteristic reactions: 40 cycles of 15 seconds at 95 °C, 1 minute at 60 °C, and 1 minute at 72 °C (Oraby et al., 2019). The collagen IV thermal cycling program; 95 °C for 2 min for polymerase enzyme activation as well as 45 cycles of denaturation (95 °C for 30 seconds) and annealing and extension (60 °C for 30 seconds) (Alomari et al., 2020). The thermal cycling program of AQP1 included 95 °C for 3 minutes for polymerase enzyme activation, followed by 40 cycles of denaturation (95 °C for 15 seconds) as well as extension and annealing (58 °C for 1 minute) (Seyahian et al., 2020). Table 1 demonstrates the investigated genes' primer sequences. The $2^{-\Delta\Delta Ct}$ method was followed for quantifying the investigated genes' mRNA expression, which was then normalized against the expression of β-actin, an endogenous control (Livak and Schmittgen, 2001).

2.10.6. Statistical analysis

The 25th version of the statistical package for social science (SPSS) (Abu-Bader, 2021) for windows was used to code and enter the data. The standard error (SE) and mean were used to express quantitative data. One-way analysis of variance (one-way ANOVA) has been utilized to examine quantitative data from more than two groups for comparisons between the researched groups. The significance level was determined at a p-value of ≤ 0.05.

Table 1. The investigated genes' primer sequences.

Genes	Primer sequences	Gene bank accession number
Vanin 1	Forward 5'- GGGAGTTTCAGGTGTTGAG-3'	XM_032896364.1
	Reverse 5'- TGAGTGTGCTATGAGGTCTG-3'	
Nephrin	Forward 5'- CCCTCCGGGACCCTACTG-3'	XM_032896170.1
	Reverse 5'- TCTGGGAGGATGGGATTGG-3'	
Collagen type IV	Forward 5'- GGACAAGCAGGCTTTCCTGGA -3'	XM_034936977.1
	Reverse 5'- TGCTGTCCAGGAAGGCCAGG -3'	
Aquaporin 1 (AQP1)	Forward 5'- GCTCAGCAGTCAAAGCCA TGT-3'	XM_032906446.1
	Reverse 5'- AAACGGAGAAGCTGGAAATGA-3'	
β-actin	Forward 5'- AGGCCAACCGTGAAAAGATG-3'	XM_032887061.1
	Reverse 5'-ACCAGAGGCATACAGGGACAA- 3'	

3. Results

3.1. Effect of Exosome, MSCs, and Balanites aegyptiacae treatments on MDA and TAC

Table 2 summarises all studied groups' MDA and TAC levels for renal tissue. MDA was markedly increased while TAC was substantially reduced in DN rats ($P< 0.001$) compared to normal health rats. The undesirable MDA elevation and TAC reduction were ameliorated in the group co-administered Balanites aegyptiacae with MSCs or exosomes compared to DN rats ($P< 0.001$).

3.2. Effect of the exosome, MSCs, and Balanites aegyptiacae treatments on TNF Alpha and insulin-like growth factor 1

Table 3 displays the results of the serum amounts of TNF-α and IGF-1 of all groups examined. DN rats demonstrated a substantial increase in TNF-α and a significant reduction ($P< 0.001$) in IGF-1 compared to normal healthy rats. Co-administration of *Balanites aegyptiaca* with exosome or MSCs substantially improved ($P< 0.001$) the undesirable alternations in TNF-α and IGF-1 than DN rats.

3.3. Histopathological examination

Histopathological analysis of H&E stained negative control kidney sections (magnification, × 400) (Figure 1A) revealed normal kidney structure with normal renal tubules and glomeruli (score lesion 0). Conversely, the kidney sections from the positive control group demonstrated a number of histological changes involving massive cortical necrosis; the necrosis and disintegration of glomerular tuft (arrow) leading to widening of the capsular spaces (*), renal corpuscles enlargement, and the renal spaces becoming narrower due to desquamation into the lumen of the tubules especially the proximal convoluted tubules, severe cytoplasmic and nuclear degeneration in the tubular epithelial cells and the glomerular hypercellularity (Figure 1B) (Score lesion ++++). Unlike Diabetic rats treated with *Balanites aegyptiaca* (Figure 1C), combined *Balanites aegyptiaca* + MSCs (Figure 1D), combined *Balanites aegyptiacae* + exosome (Figure 1E), MSCs (Figure 1F), and exosome (Figure 1G) exhibited ameliorated in kidney structure as illustrated.

3.4. Effect of the exosome, MSCs, and Balanites aegyptiacae treatments on the relative expression of vanin-1 as well as Nephrin mRNA in kidney tissue

Figure 2 demonstrates the relative mRNA expression of vanin-1 and nephrin in renal tissue across all groups investigated. Vanin-1 and Nephrin mRNA expression in DN rats was substantially increased compared to the normal health rats. Upregulation of Vanin-1 and Nephrin

Table 2. Effect of exosome, MSCs, and *Balanites aegyptiacae* treatments on MDA and TAC in renal tissue of all examined groups.

Groups	Malondialdehyde (nmol/g tissue)	% change	Total antioxidant capacity (mM/L)	% change	P Value
Negative control group	199.6±1.1^{c}	---	1.73±0.00^{c}	---	< 0.001
Positive control group	284.6±2***	42.58%	0.200±0.02***	-88.43%	
Balanites treated group	202.8±0.6^{c}	-28.74%	0.99±0.01^{***c}	395%	
MSCS treated group	199±2.1^{c}	-30.07%	0.998±0.00^{***c}	399%	
Exosome-treated group	232.2±0.53^{***c}	-18.41%	1.004±0.00^{***c}	402%	
Balanites + MSCs treated group	234.8±2.1^{***c}	-17.49%	1.024±0.00^{***c}	412%	
Balanites + exosomes treated group	196.4±0.65^{c}	-30.99%	1.058±0.00^{***c}	429%	

Note: Values are presented as mean ± SEM, n = 10. Significant differences ($P<0.05$) are represented by different superscript letters for values. $^{c}P< 0.001$ compared to positive control group. % change of positive control was calculated according to negative controls. $^{***}P< 0.001$ compared to control group.

Table 3. Effect of the exosome, Mesenchymal stem cells, and *Balanites aegyptiacae* treatments on serum TNF-α and IGF-1 levels among all examined groups.

Groups	TNF-α (pg/ml)	% change	IGF-1 (Pg/ml)	% change	P Value
Negative control group	4.94±0.24^{c}	---	208421.25± 2129.2^{c}	---	< 0.001
Positive control group	7.44±0.07***	50.60%	116555± 1932.7***	-44.07%	
Balanites treated group	5.98±0.19^{c}	-19.62%	172131.25± 2807.9^{***c}	47.68%	
MSCS treated group	6.65±0.07^{*c}	-10.61%	162562.5± 914.4^{***c}	39.47%	
Exosome-treated group	5.31±0.23^{c}	-28.62%	187055± 3127.0^{***c}	60.48%	
Balanites + MSCs treated group	5.35±0.08^{c}	-28.09%	180486± 2649.6^{***c}	54.85%	
Balanites + exosomes treated group	3.76±0.24^{c}	-49.46%	206096± 914.4^{c}	76.82%	

Note: Values are presented as mean ± SEM, n = 10. Significant differences ($P<0.05$) are represented by different superscript letters for values, $^{c}P< 0.001$ compared to positive control group. % change of positive control was calculated according to negative control. $^{*}P< 0.05$ compared to the control group. $^{***}P< 0.001$ compared to the control group.

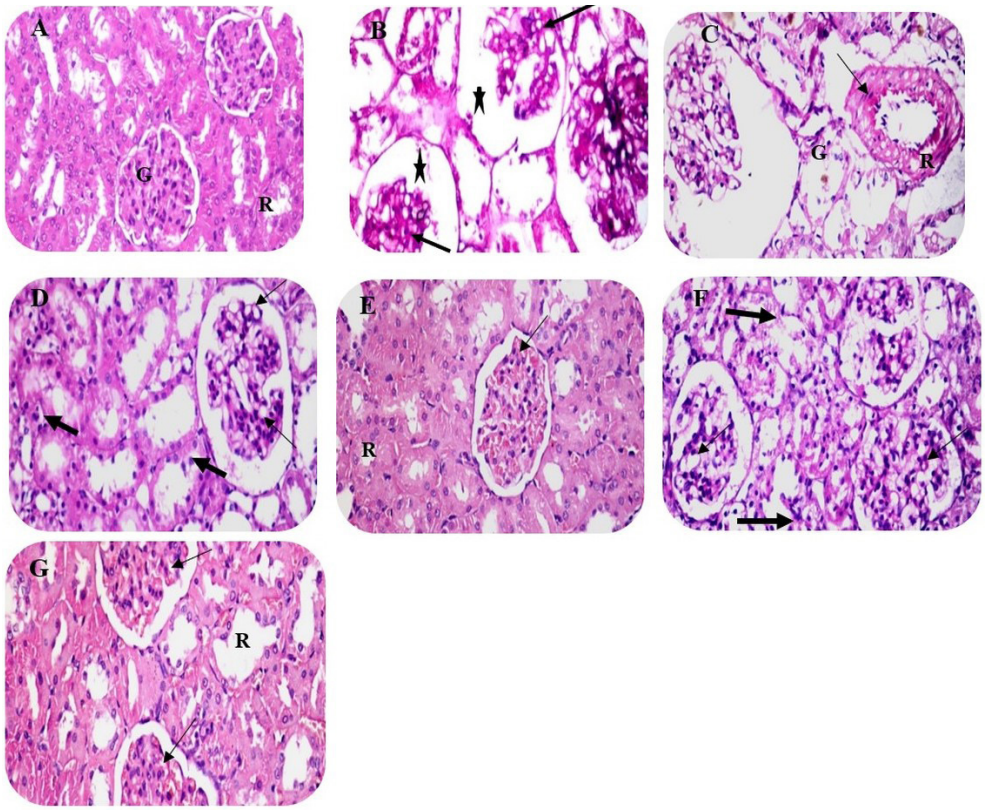

Figure 1. Photomicrographs of kidney tissue from all groups studied: (A) The kidney structure in the negative control group was normal, with normal glomeruli and renal tubules (score lesion 0). (B) Kidney section from the positive control group demonstrated several histological alterations, including massive cortical necrosis, the necrosis, and disintegration of glomerular tuft (arrow), leading to widening of the capsular spaces (*). (C) Kidney section from *the Balanites aegyptiaca* treated group displayed cortical vacuolar degeneration in the renal glomeruli and renal tubules together with thickening and dilating of the muscular wall of the interstitial blood vessel (arrow) (Score lesion +++). (D) The kidney section from *the Balanites aegyptiacae* + MSCs treated group exhibited mild cortical degeneration; note the vacuolar degeneration in the glomerular epithelium (thin arrows) and the renal tubular epithelium (thick arrows), (Score lesion ++). (E) The kidney section from *Balanites aegyptiacae* + exosome exhibited slight glomerular congestion (arrow), the renal tubules (R) indicating normal basement membrane and lining epithelium (Score lesion +). (F) Kidney section from MSCs treated group mild cortical degeneration; note the vacuolar degeneration in the glomerular epithelium (thin arrows) and the renal tubular epithelium (thick arrows) (Score lesion +++). (G) The kidney section from exosome treated group demonstrated mild glomerular congestion (arrow), and the renal tubules (R) indicated normal basement membrane and lining epithelium (HE X400) (Score lesion +).

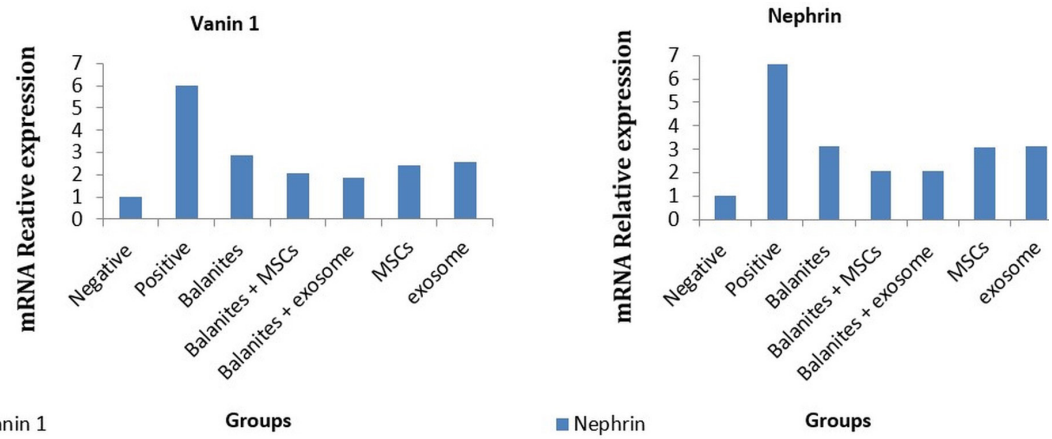

Figure 2. Effect of *Balanites aegyptiacae*, MSCs and exosome on renal vanin-1 and Nephrin mRNA expression. The values are calculated in (mean ± SEM).

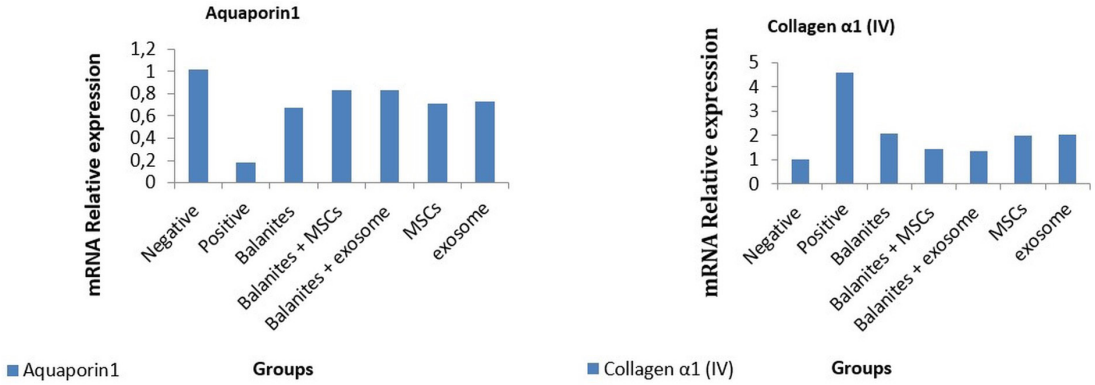

Figure 3. Effect of *Balanites aegyptiacae*, MSCs and exosome on renal aquaporin-1 (AQP1) and collagen IV mRNA expression. Values are calculated in (mean ± SEM).

was significantly lowered following administration of *Balanites aegyptiacae, Balanites aegyptiacae* + MSCs, *Balanites aegyptiacae* + exosome, MSCs and exosome by -52.5%, -65.83%, -69.08%, -60% and -57.5% respectively in Vanin-1 gene expression in addition, -52.63%,-68.87%,-68.87%, -53.75 and -52.78% in Nephrin mRNA expression as compared to DN rats ($P< 0.001$).

3.5. Effect of the exosome, MSCs, and Balanites aegyptiacae treatments on mRNA relative expression of collagen IV and aquaporin-1 (AQP1) in kidney tissue

According to Figure 3, the treatment of Exosome, MSCs, and Balanites aegyptiacae significantly reduced collagen IV mRNA in DN rats. These findings revealed a substantially lower proximal nephron water channel (AQP1) expression in DN rats compared to normal healthy rats. Moreover, a considerable rise in AQP1 mRNA expression was noticed in the groups co-administered *Balanites aegyptiacae* with MSCs or exosomes compared to DN rats.

4. Discussion

Diabetes mellitus has significant microvascular disorders, including DN (Fan et al., 2019). End-stage renal disease (ESRD) is detected in approximately 40% of cases in diabetic patients (Kundu et al., 2020). DN is a chronic disorder characterized by proteinuria, impaired kidney function due to high sugar levels, glomerular hypertrophy, reduced glomerular filtration, and renal fibrosis (Sun et al., 2013).

Streptozotocin is a drug that promotes specific cytotoxicity in pancreatic islet β- cells and is commonly utilized to induce diabetes mellitus in animal models (Aziz et al., 2020). Protein carbamoylation, DNA alkylation, ROS, RNS free radicals production, and O-GlcNAcse inhibition are all reasons for β-cell toxicity (Islam et al., 2017). Nicotinamide/Streptozotocin-induced diabetes mellitus was chosen as a type 2 diabetes mellitus model to study the *Balanites aegyptiacae* aqueous fruit extracts, MSCs, and exosome effects. When NIC is administered before the injection of streptozotocin in this model, the DM severity is significantly reduced, resulting in a T2DM-like syndrome with impaired insulin sensitivity (Aziz et al., 2020).

Novel therapeutic strategies for DN prevention are urgently needed (Tanaka et al., 2012). Crude extracts or purified molecules from medicinal plants have been used to treat several disorders because they serve as a reservoir of highly potent ingredients (Zaky et al., 2022). *Balanites aegyptiacae*, a medicinal plant, is widely distributed in Africa. Mesocarp of its fruit is extensively utilised as an oral antidiabetic medication (Gnoula et al., 2008) in Egyptian folk medicine, demonstrating improved diabetic complications toward normal in diabetic-induced animals (Al-Malki et al., 2015).

MSCs are critical immunological modulators with potential therapeutic benefits (Portha et al., 2011). There is growing proof that MSCs possess various therapeutic benefits that depend not only on their capacity for differentiation to repair injured tissue but also on their capacity to regulate and stimulate progenitor endogenous cells (Hsiao et al., 2015). Since nephrons, essentially mesenchymal in origin as well as stromal cells, are critical for signalling that results in the development of both collecting ducts and nephrons, MSCs are potential candidates for kidney repair (Anglani et al., 2002).

Exosomes, which are microvesicles produced from MSCs, have recently been used commonly in experimental studies, and it has been demonstrated that they help MSCs exert their therapeutic benefits (Katsuda et al., 2013; Yu et al., 2014). Cell culture supernatants of MSCs have been reported to contain exosomes. Exosomes are microvesicles that include microRNAs (miRNAs), mRNAs, DNA, and proteins (Valadi et al., 2007), mediating information exchange between cells. Although exosomes have been shown to be effective in treating acute renal disease (Tomasoni et al., 2013; Zhou et al., 2013; Morigi and Coppi, 2014) the efficacy of exosomes extracted from the bone marrow of MSCs in treating DN is still to be determined.

The current study aims to investigate the potential hypoglycemic effect and the mode of action of the aqueous *Balanites aegyptiacae* fruits extracts in DN. Furthermore, this research assesses exosomes in treating diabetes

complications, which is believed to become a unique strategy for treating diabetic complications.

Moreover, hyperglycemia causes oxidative stress (Idris et al., 2001), one of the most fundamental reasons for chronic complications of T2DM. It leads to the production of several oxidative intermediates, resulting in oxidative damage (Cheng et al., 2019). Malondialdehyde is a secondary byproduct of lipid peroxidation that could be employed as a biomarker to evaluate levels of oxidative stress. It is found to be higher in disorders like diabetes that lead to oxidative stress (Tripathi et al., 2016). Multiple explanations have been suggested to illustrate why oxidative stress is elevated in DM, and these explanations can be divided into two principal categories: higher ROS production and lowered antioxidant defense mechanism (Hisalkar et al., 2012).

These findings revealed that renal MDA levels of the DN rats were substantially higher than those of normal health rats. These findings concur with earlier research (Ali et al., 2017), which revealed that induction of DN resulted in a substantial rise in renal MDA levels compared to the control group. While administration with *Balanites aegyptiacae*, MSCs and exosomes led to a considerable decrease in MDA levels in comparison to DN rats ($P< 0.001$). These findings concur with earlier research (Qusti et al., 2015) which revealed that injecting *Balanites aegyptiacae* into rats protects them against streptozotocin-induced oxidative kidney injury. *Balanites aegyptiacae* active ingredient composition includes furanocoumarin, saponin, and flavonoids such as quercetin 3-glucoside, 3-glucoside, and 3-7-diglucoside, which was shown to have the capacity to neutralized free radicals (Chothani and Vaghasiya, 2011). MSCs have been shown to have antioxidant and anti-apoptotic effects (Peired et al., 2016) by transferring their secretome and mitochondria to nearby, in-contact injured cells (Bochon et al., 2019). Mesenchymal stem cells trigger the expression of antioxidant enzymes, reduce ROS generation, and participate in mitochondrial repair (Lee et al., 2019) and cell death inhibition (Crigna et al., 2018).

The renal TAC of DN rats was found to be substantially lower than that of normal health rats. These findings concur with earlier research (Jiang et al., 2015) which illustrated that diabetes mellitus was related to reduced antioxidant potential in diabetic animals' blood and tissues. Groups treated with *Balanites aegyptiacae*, MSCs and exosomes demonstrated a substantial increase in TAC levels in comparison to DN rats. These findings concur with earlier research (Hassanin et al., 2018) that illustrated that *Balanites aegyptiacae* had hypoglycemic, hypolipidemic, and insulinotropic effects in pancreatic β-cells, which are associated with oxidative stress reduction, antioxidant defense system increase, and reduced apoptosis. The antioxidant activities of *Balanites aegyptiacae* fruit were believed to result from an increase in the enzyme synthesis via regulating components of the antioxidant response in the enhancer region of the enzyme-coding gene's promoter site (Ayoola et al., 2011 ; Khalil et al., 2016).

Hyperglycaemia that contributes significantly to the aetiology of DN causes mitochondrial ROS generation, glycation of scavenging enzymes, followed by renal dysfunction and cell death (Lee et al., 2003). Inflammation is closely associated with the DN progression besides oxidative stress. According to the findings, TNF-α, which is synthesized intrinsically in cells of the kidney, is one of the primary pro-inflammatory cytokines in the development of renal damage (Navarro et al., 2005 ; Kalantarinia et al., 2003 ; Moresco et al., 2013). Furthermore, increased oxidative stress can enhance the production of inflammatory cytokines, enhancing the generation of free radicals (Elmarakby and Sullivan, 2012).

The present study showed a substantial elevation in TNF-α levels for DN rats compared to normal healthy rats. In comparison to DN rats, co-administration of *Balanites aegyptiacae* with MSCs and exosomes significantly improved the undesired alterations values in TNF-α. These findings align with earlier research (Elkareem et al., 2021 ; Gomaa et al., 2022) that indicated that the anti-inflammatory activities of *Balanites aegyptiaca* aqueous extract were because of its inhibition of the production of the TNF-α and IL-1β inflammatory mediators, as well as its antioxidant characteristics. In addition, these results support those from an earlier study (Al-Malki et al., 2015) indicating that TNF-α, IL-1β, and VEGF were substantially lowered in diabetic rats that received ethyl acetate extract of *Balanites aegyptiacae* compared to untreated diabetic rats. Hence, the mechanism of cytokines-inhibitory effects of *Balanites aegyptiacae* aqueous extract may include radical scavenging. The high amount of β – sitosterol, vanillic acid, and syringic acid, which represent scavengers for free radical and trigger antioxidant potential, was found to be responsible for the antioxidant potential of *Balanites aegyptiacae* aqueous extract (Sarker et al., 2000).

MSCs are essential for tissue repair and regeneration, generating local healing signals and anti-inflammatory (Huang et al., 2017 ; Wu et al., 2020). The action of MSCs can be attributed to several mechanisms, including a primary mechanism involving replacing damaged cells through differentiation and their paracrine features. MSCs create chemokines, growth factors, and cytokines, which aid in the regeneration and protection of other cells while stimulating their proliferation and differentiation (Kim et al., 2005 ; Liu et al., 2013 ; Zimmerlin et al., 2013 ; Guan et al., 2014 ; Bai et al., 2015 ; Spees et al., 2016 ; Dabrowska et al., 2018). These results are supported by another study (Nassar et al., 2016), where MSC-EVs also have been tested in individuals with chronic kidney disease to evaluate the therapeutic potential and safety of MSC-EVs extracted from the umbilical cord in slowing the progression of CKD. Furthermore, patients with CKD treated with MSC-EVs had a different inflammatory immune response, as evidenced by significant increases in IL-10 and TGF- β1 and a reduction in TNF-α in plasma. Increasing evidence suggests that the MSC-Exos miRNAs are involved in suppressing inflammatory progression. By providing recipient cells with organelles, lipids, microRNA (miRNA), mRNA, proteins/peptides, and DNA, Exos isolated from MSC could replicate the parental MSCs function (Baglio et al., 2015). Because there is no risk of immunological rejection or aneuploidy, MSC-derived exosomes perform a vital role in exerting therapeutic effects. Exosomes may therefore be an ideal alternative therapy for various disorders (Yu et al., 2014).

Insulin-like growth factor-1 is a multipotent growth factor that controls metabolism and tissue growth. Furthermore, due to its reduction of glucose and insulin-sensitizing effects, it has been hypothesized that IGF-1 improves glucose homeostasis (Moschos and Mantzoros, 2002 ; Frystyk, 2004). IGF-1 can potentially increase glucose and fatty acid absorption in peripheral tissues (Giustina et al., 2015). Our results revealed that serum levels of IGF-1 in DN rats are considerably less ($P< 0.001$) than in normal health rats. These results are in accordance with prior studies (Moxley III et al., 1990 ; Spagnoli et al., 1999), which suggested that poor glycemic control in diabetes is related to lower blood IGF-I levels. The primary source of IGF-1 circulating in the blood is considered to be the liver. Decreased total and free IGF-1 levels in serum might be due to decreased IGF-1 production in the liver (Heo et al., 2000). Furthermore, insulin insufficiency or resistance may be the primary cause of IGF-1 gene down-regulation (Kaytor et al., 2001 ; Li et al., 2004). Administration with *Balanites aegyptiacae*, MSCs, and exosomes demonstrated a significant rise in serum IGF-1 levels compared to DN rats. Insulinomimetic activities (Motaal et al., 2012), insulin secretion stimulation, and potentiation increased insulin receptors affinity (Abde-Moneim, 1998), ameliorated hepatic glycogen concentration, accelerated glucose metabolism, decreased intestinal glucosidase production, and reduced liver gluconeogenesis (Gad et al., 2006) could all be linked to the ameliorative effects of *Balanites aegyptiacae* extracts. Furthermore, we could suggest that these actions may be contributed to the elevation of serum IGF-1 levels in groups administered with *Balanites aegyptiacae* aqueous extract.

MSCs have been linked to IGF-1 release. The pro-regenerative effects of MSCs genetically modified to mute IGF-1 expression were limited (Imberti et al., 2007). These results are confirmed by prior studies (Kinnaird et al., 2004 ; Nagaya et al., 2005), which indicated that mesenchymal stem cells could release IGF-1 in vitro studies. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway that is implicated in the progression of cell survival and cell cycle is crucial for IGF-1's mitogenic action on other cells (Liu and Zhang, 2011 ; Sandri et al., 2013). Cell culture supernatants of MSCs have been known to contain exosomes. Exosomes include microRNAs (miRNAs), DNA, mRNAs, and proteins (Valadi et al., 2007), contributing to cell-to-cell communication. As a result, we concentrated on the exosome role as a crucial factor in MSC paracrine actions in DN; nevertheless, exosome effects on acute renal disease have been documented (Tomasoni et al., 2013; Zhou et al., 2013; Morigi and Coppi, 2014).

According to the histopathological investigation of the kidney sections, the kidney structure of negative control was normal, with normal renal tubules and glomeruli (Figure 1A). Conversely, renal sections of DN rats revealed multiple histopathological changes involving massive cortical necrosis; the necrosis and disintegration of glomerular tuft leading to widening of the capsular spaces, Renal corpuscles expansion, and renal spaces becoming narrower (Figure 1B). However, these histopathological changes observed in our study were significantly improved in rats co-administered Balanites with either MSCs or exosome, with more improvement in the exosome group showing mild glomerular congestion, normal basement membrane and lining epithelium of the renal tubules (Figure 1C, 1D, 1E, 1F, and 1G). These findings are consistent with those of an earlier investigation (Nagaishi et al., 2016) which proved that exosome injection improved the histological image of DN with the help of reduced degeneration, vacuolation, atrophic alterations, and inflammatory cell infiltration of proximal tubule epithelial cells (PTEC), as verified by H and E staining. Additionally, it was reported that MSCs have the ability to control the immunological environment, decreasing fibrosis and increasing angiogenesis. Furthermore, exosome-mediated paracrine action is involved in most of these processes, implying that exosomes are essential in the recovery of kidney function (Nagaishi et al., 2016). Another study (Nargesi et al., 2017) suggested that renal structure and function can be substantially preserved using MSC-derived EVs. However, in animal models, only one clinical study has investigated the renoprotective actions of EVs generated from MSCs on chronic kidney disease development.

Vanin-1 is a pantetheinase-active epithelial ectoenzyme. It plays several roles, promoting the conversion of pantetheine to pantothenic acid (vitamin B5), an essential precursor in forming coenzyme A, and inflammation and oxidative stress. It is abundant in the liver, intestine, and kidneys (Bartucci et al., 2019). Nephrin, a transmembrane protein, has been described as a structural protein of functional significance in preserving the glomerular filtration barrier of the podocyte slit diaphragm (Ruotsalainen et al., 1999) as well decreased levels are strongly linked to podocyte mass loss (Aaltonen et al., 2001). Compared to normal health rats, the relative mRNA expression of Vanin-1 and Nephrin was considerably higher in DN rats. These findings concur with those of an earlier investigation, (Oraby et al., 2019) which found that in untreated fructose-STZ rats, the expression of Vanin-1 was much higher than in non-diabetic rats. In fact, during diabetes circumstances, the expression of vanin-1 increases, Vanin-1 is believed to produce cysteamine, which is considered vital to oxidative stress and inflammatory responses (Hosohata et al., 2014) that suppress the antioxidant activities such as GSH and enable free radicals to damage tissue through oxidation (Saghaei et al., 2012). Another study (Sung et al., 2006) suggested that nephrin mRNA expression levels upregulated in mice diabetic kidneys.

Multiple alterations in the cells responsible for maintaining the glomerular basement membrane have been observed in the DN mechanisms (Ha and Kim, 1999). Increased fibronectin, transforming growth factor (TGF), collagen type IV synthesis, and altered Na+ -K+-ATPase function are among the alterations (Koya et al., 1997 ; Hoffman et al., 1998). Interestingly, the alterations in the glomerular basement membrane, involving a rise in fibronectin and collagen type IV, may result from nonenzymatic glycosylation, elevation in reactive oxygen species, and hyperglycemia (Aaltonen et al., 2001). These alterations are linked to the protein kinase C activation that is believed to cause several symptoms of

DN (Scivittaro et al., 2000). In cultured cells that express nephrin, protein kinase C activation results in a notable rise in nephrin mRNA and protein synthesis. Nephrin is linked to the early phases of permeability barrier disruption in DN. The functional alterations were accompanied by elevated mRNA levels and altered nephrin localization patterns within the glomerulus (Aaltonen et al., 2001).

Our results revealed that both Vanin-1 and Nephrin mRNA expression in the kidney was markedly decreased after co-administration of Balanites aegyptiacae with MSCs or exosomes compared to the positive control group, which is another evidence for ameliorating early DN.

In renal tissue, collagen IV is a significant glomerular extracellular matrix (ECM) in the glomerular mesangial areas (Ha et al., 2009). Our results demonstrated a significant rise in relative collagen IV mRNA expression in DN rats compared to normal healthy rats. These findings are consistent with an earlier investigation (Killen et al., 1987) that found upregulation in mRNA of collagen type IV in the kidneys of streptozotocin-induced diabetes in mice. Furthermore, it has been revealed that diabetes is associated with an elevation in renal mRNA collagen-IV expression (Alomari et al., 2021). The reasons for collagen IV upregulation in the renal DN involve: firstly, hyperglycemia can promote the production of collagen IV and its stability. Second, nonenzymatic protein glycosylation suppresses collagen IV crosslinking, which increases collagen IV stability and decreases its degradation (Cohen et al., 2007). However, groups treated with co-administration of *Balanites aegyptiacae* with MSCs or exosomes exhibited a substantial decrease in relative collagen IV mRNA expression in the renal tissue in comparison to DN rats. These findings are compatible with those of an earlier investigation (Xiang et al., 2020), which proved that collagen IV, α-SMA, and TGF-β as inflammatory markers in renal DN rats were reduced following the MSCs treatment indicating that MSCs can suppress fibrosis. Another study (Lv et al., 2013) demonstrated that MSCs had a reno-protective effect by reducing inflammation and fibrosis, and MSCs have been considered renotropic (Morigi et al., 2004 ; Park et al., 2012).

Extracellular vesicles (EVs) produced from MSCs have an anti-fibrotic mechanism that involves miRNA targeting fibrosis-related genes (Grange et al., 2019a ; Grange et al., 2019b ; Zhang et al., 2020). MSC-exosomes, For example, delivered miRNA-let7c to mouse tubular epithelial cells with unilateral ureteral obstruction, which suppressed the production of TGF-β1, α-SMA, metalloproteinase-9 (MMP9), as well as collagen IVα1 and its receptor (Wang et al., 2016).

Several kidney disorders, including DN, have been linked to dysregulation of "aquaporin; AQP," which is the water channel membrane protein in the kidney (He and Yang, 2019). AQP-1 is widely expressed on proximal tubular cells and the descending limb of Henle's loop cells (Kortenoeven and Fenton, 2014) and participates in hypertonicity formation (Lanaspa et al., 2010). It was also found in the glomerular basement membrane's β-laminin (Bedford et al., 2003). Furthermore, groups co-administered *Balanites aegyptiacae* with MSCs or exosomes exhibited a substantial increase in AQP1 mRNA expression ($P<0.001$) compared to DN rats.

These findings align with an earlier investigation (Ismail et al., 2016) which found that diabetes significantly downregulated AQP1 expressions in renal tissues compared with control. Another study also (Nielsen et al., 2002) suggested that the lower aquaporins levels in renal tubules could influence nephron water management and represent the development of acute kidney injury. Furthermore, the decline in AQP1 expression could be caused by increased protein degradation (Hassouneh et al., 2016).

5. Conclusion

The current research revealed that the co-administration of exosomes isolated from MSCs preconditioned with *Balanites aegyptiacae* gave better therapeutic effect on DN in rats than treatment with MSCs or exosomes alone. This beneficial effect of exosomes was mediated, at least partially, through inhibiting inflammation, apoptosis, and oxidative stress while inducing antioxidant status, regeneration, and angiogenesis, resulting in better renal repair and function. This exosome-based treatment method could be beneficial in treating DN. Future clinical trials will be required to assess the safety and clinical utility of this therapeutic approach for patients.

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