MANSOURA JOURNAL OF CHEMISTRY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-4938

Possible Ameliorative Mesenchymal Stem Cells -Based Therapy of a Bladder – Outlet –Obstruction-Induced Overactive Bladder

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Received: 26/05/2024
Accepted: 28/07/2024

Abstract: Purpose: This study's objective was to assess the possible therapeutic and ameliorative effect of adipose derived mesenchymal stem cells (ADMSCs) on the overactive bladder (OAB) induced by bladder outlet obstruction (BOO) through the evaluation of some of oxidative stress, and anti-apoptotic markers in the bladder tissue, in addition to histopathological examinations.

Methods: Regarding this investigation, Fifty female Sprague-Dawley mature rats with age (6 weeks) weighing 180 ± 20 gm. were divided into four groups at random as the following: normal control group (n=10), OAB group (n=20) and OAB+ADMSCs group (n=20): After 4 weeks of BOO, $1x10^6$ of ADMSCs were once directly injected into the bladder's anterior wall's sub-mucosal layer .On the day 28 after the injection of ADMSCs, the scarifying of the animals were done and the bladder samples were collected from all rats groups. Catalase (CAT) activity and malondialdehyde (MDA) were measured in bladder tissue homogenates from all rats groups as oxidative stress markers. Also, gene expression of both apoptotic marker B-cell lymphoma 2 (Bcl-2) of bladder tissue of all rats groups was determined. Furthermore, histopathological changes in the bladder mucosa were detected in all different rats groups.

Results: OAB+ADMSCs group showed statically significant higher expression ($p<.001$) of antioxidant marker (catalase) when compared by OAB group and totally inversed with the malondialdehyde that show statically significant lower expression ($p<.001$) when compared with OAB group. There was a significant increase ($p<.001$) in the anti-apoptotic markers B-cell lymphoma 2 (BCL2) in comparison to OAB group .The bladder's structure of overactive rats which treated with ADMSCs significantly improved.

Conclusions: The results manifested the effective role of ADMSCs in enhancing the recovery of the bladder after the induction of OAB.

Keywords: MSCs, Over active bladder, Bladder Outlet Obstruction.

1.Introduction

The chronic condition known as overactive bladder (OAB) is characterized by urgency along with increased frequency, nocturia, and urgent urine incontinence. The primarily cause of OAB is detrusor over activity. Detrusor over activity is the term used to describe involuntary contractions of the detrusor muscle that occur during the bladder filling phase as a result of persistently elevated bladder afferent activity [1].

There are three basic theories for detrusor over activity occurring, myogenic (e.g., bladder

outlet obstruction (BOO), neurogenic (e.g., spinal cord injury) and inflammatory (e.g., interstitial cystitis) or idiopathic. [2].

The common disorders that result in BOO arise from congenital anomalies, such as bladder neck sclerosis, posterior urethral valve, urethral stricture and benign prostatic hyperplasia (BPH) [3, 4].

The current treatment of OAB is based on three tires are as follow: First tire lasts for six weeks and involves 2-3 sessions of bladder training. Second tire, is medical therapy, Anticholinergic (anti muscarinic) medication is the cornerstone of treatment for overactive bladder (OAB). Despite more than 60% of these medications, have negative side effects. Dry lips, dry eyes, impaired vision, and constipation can also affect the CNS, resulting in drowsiness, disorientation, and a decline in cognitive ability. The tertiary tire, is to treat and control the aforementioned voiding issues

using surgical techniques such as neuromodulation of the posterior tibial nerve or sacral nerve 3 (S3).This should to be minimally invasive, easy to use, not embarrass the patient's private area, durable, and economical [5].

To provide a long-term/stable therapeutic cure for OAB, a novel physiological and therapeutic approach must be created [4]. Many bladder problems can be treated with stem cell therapy, which is thought to be a revolutionary therapeutic strategy. Mesenchymal stem cells (MSCs), which are present in adult bone marrow, adipose tissue, and cord blood, have in fact produced encouraging outcomes in preclinical research on stress urinary incontinence, OAB, detrusor under activity, and injury to the bladder and urethra [6, 7].

Bladder dysfunction caused by closure of the bladder outflow is largely attributed to oxidative stress[8, 9]. When the production of reactive oxygen species (ROS) are not in balance with the ability of biological systems to repair oxidative damage or neutralize the effects of reactive intermediates including peroxides and free radicals, Oxidative stress occurs typically in that such cellular environment. High levels of (ROS) impair antioxidant defence mechanisms causing protein, lipid, and DNA damage which in turn disrupts cellular processes and ultimately leads to cell death.

The current study aims to evaluate the therapeutic effect of ADMSCs in treating overactive bladder depending on their paracrine effect throughout reducing oxidative stress and preventing cellular apoptosis.

2.Materials and methods

2.1.In vitro study:

2.1.1.Isolation and Expansion of adipose-derived mesenchymal stem cells (ADMSCs)

Para gonadal fats of Sprague-Dawley rat testis were isolated, chopped, and digested with 0.075% type I Collagenase (Invitrogen, US) at 37°C for 1 hour. Then centrifugation for 5 min at 600 g was applied. Cellular pellets were cultured in DMEM media with 10% fetal bovine serum (FBS) and streptomycin (100 µg/ml) (Gibco, Thermo, USA) in an incubator with 5% carbon dioxide at 37°C. When the cells reached 70%–80% confluence, they were trypsinized and sub-cultured, and cells at passages three were used in the subsequent experiments.

2.1.2.Characterization of ADMSCs by flow cytometry:

100 µl from re suspended ADMSCs cells was taken and incubated with antibodies that conjugated with different fluorescent probes (Becton, Dickinson) PE-conjugated CD44, FITC conjugated CD45 at room temperature for 30 min in the dark. After that, this mixture was wash with 2 ml of stain buffer and centrifuged for 10 min at 2000 rpm. Discard the supernatant, and then dissolve the pellet with 500 µl of stain buffer. The labeled cells were identified by BD Accuri C6 flow cytometry and the result analysis by Flow JO BD software.

2.1.3.Experimental Animals

Fifty mature female Sprague-Dawley rats weighing 180 ± 20 g and aged 8-10 weeks were kept at a density of 4 rats for each polycarbonate cage. These animals were put in a regulated with a 12 hour light/dark cycle, 24^{o}C air conditioning, and 50-70 % relative humidity. Food and water were available *ad libitum* throughout the experiment. All care and procedures adapted for the present study were according to the NIH guide, and animals used the approval of the Institutional Animal Ethics

Committee of the Faculty of Science, Mansoura University MU-ACUC (SC.MS.23.03.20).

2.2. Animal groups

Fifty female rats were randomly divided into three groups as following: control group (n=10), OAB group (n=20): this group underwent to BOO operation, and the last group was OAB+ADMSCs group (n=20): this group underwent to BOO operation and treated with ADMSCs. After 4 weeks of BOO, the anterior wall of the bladder's sub-mucosal layer received a single direct injection of $1x10^{6}$ of ADMSCs. At the end of experiment (on the day 28 after the injection of ADMSCs), the animals were sacrificed.

2.2.1. Partial BOO animal model:

All animal experiments were approved by Laboratory animals (ILAR 1996). All protocols were submitted by the ethical committee of Mansoura University, Urology and Nephrology Center. Partial BOO was induced in 6-week-old female Sprague Dawley rats through urethral ligation. A section of polyethylene 10 tubing was utilized to catheterize the bladder transurethrally. The peritoneal cavity was accessed by making a suprapubic incision. The catheterized urethra was located near the neck of the bladder, minimally dissected, and sutured with a 4-zero sutures. The abdominal incision was closed and the catheter was removed once the knot was tightened.

2.3.Collection of tissue samples:

At the end of the experiment, Cervical dislocation was used to sacrifice the animals.

A midline laparotomy was done, and The bladder of each rat was divided longitudinally into three parts, one was preserved at -80℃ for oxidative stress markers measurements and another one preserved in 1 ml RNA latter at -80℃ for gene expression of anti-apoptotic marker (BCL-2) and The left was kept for bladder histopathology analyses in 10% buffered formalin.

2.4. Evaluation of oxidative stress status:

The tissue from the bladder was chopped, and blended. Following centrifugation of the homogenate for 15 minutes at 4000 rpm, the supernatants were retained for the commercial colorimetric kit (Bio-Diagnostics, Giza, Egypt) test of catalase (CAT) activity and the measurement of malondialdehyde (MDA) as a biomarker of oxidative stress.

Bladder tissue was weighed, minced, and homogenized. Then homogenates were centrifuged at 4000 rpm for 15 minutes and the supernatants were kept for the assay of catalase (CAT) activity and the determination of malondialdehyde (MDA) as indicator of oxidative stress using the commercial colorimetric kit (Bio-Diagnostics, Giza, Egypt).

2.5. Gene expression of BCL-2 in bladder tissue homogenate:

The impact of ADMSCs on apoptotic markers of bladder was assessed by measuring the gene expression of anti-apoptotic marker [BCL-2] the primer list was shown in (Table 1). In summary, tissue samples from each group were used to isolate RNA, which was then followed by cDNA synthesis and the RT-PCR reaction, which was carried out as previously stated [10].

Table (1): The primer sequence utilized for gene expression.

Genes	Sequence (5'-3')
BCL2	F: GGTGAACTGGGGGAGGATTG
	R: GCATGCTGGGGCCATATAGT
GAPDH	F: AGACAGCCGCATCTTCTTGTR: TTCCCATTCTCAGCCTTGAC

2.6. Histopathological examination:

The bladder tissues were embedded in paraffin and fixed in buffered formalin. Hematoxylin and eosin (H&E) was used to stain paraffin sections that had a thickness of five μm. Lastly, they were dried, tallied, covered, slid, and then examined by the light-microscope. (Olympus, Japan) [11 , 12].

2.7. Statistical analysis:

Normally distributed variables have existed as mean ± SD. The significance between two comparisons was detected by student t-test, while significance between multiple comparisons was carried out using one-way ANOVA and post hoc test. The SPSS program v20 (IBM Corp., USA) performed a correlation between parameters. Statistical significance was considered for p-value ≤0.05.

3. Results and Discussion

3.1. Characteristics of ADMSCs:

After 10-12 days, the ADMSCs reached 70 – 80 % confluence with long-spindle-shaped fibroblast-like cells, as shown in Fig. 1A. The phenotypic characterization showed that the isolated ADMSCs showed that the expression of surface antigens of AD-MSCs were negative for CD45 (92%) and positive for CD44 (68.9%) (Fig.1).

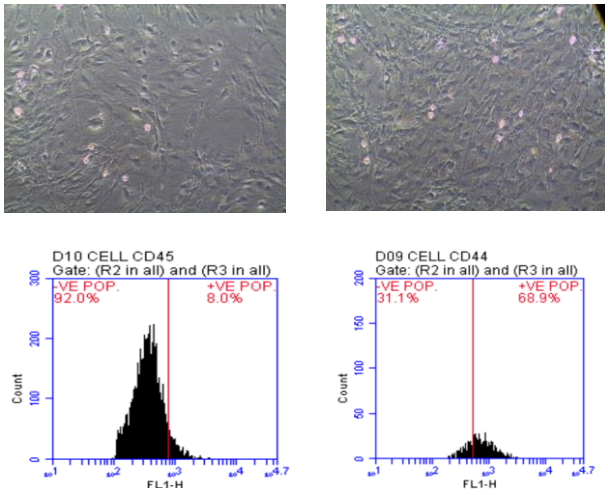

Figure (1): A) ADMSCs after 5 days from isolation, B) ADMSCs at passage 3, C) Cells acquired positive for CD44 (68.9%), and D) negative expression of CD45 (92%).

2.8.Evaluation of bladder tissue oxidative stress markers:

The content of MDA was markedly increased ($p<0.001$) within the OAB rats group when compared with the corresponding values of the control group ($p<0.001$), indicating bladder damage. In contrast, treatment of the OAB rats group with AD-MSCs exhibited an amelioration in oxidative stress by showing a highly significant decline ($p<0.001$) in MDA content values compared to that of the OAB group but are still significantly higher ($p< 0.001$) than that of the control group. On the other hand, OAB group showed a highly significant reduction ($p< 0.001$) in CAT activity values compared with the corresponding values in the control rat group. Meanwhile, the OAB treated with ADMSCs group revealed a highly significant elevation ($P<0.001$) in the values of CAT activity when compared to that of the OAB group, but are still markedly ($P<0.001$) lower than that of the control healthy rats group. (Table.2 and figures 2, 3).

Table (2): CAT and MDA levels of the studied groups

Group / Variable	Control	OAB	ADMSCs
MDA level (nmol /g tissue)	19.12 ± 0.82	31.38 ± 1.44[a]	23.57 ± 0.99[ab]
CAT (U/g tissue)	21.88 ± 1.05	11.75 ± 0.73[a]	16.15 ± 1.34[ab]

Data are expressed as mean ± SE.

Significant difference compared to corresponding [a]control, and [b]OAB group by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons (Scheffé test) at $p\leq0.05$.

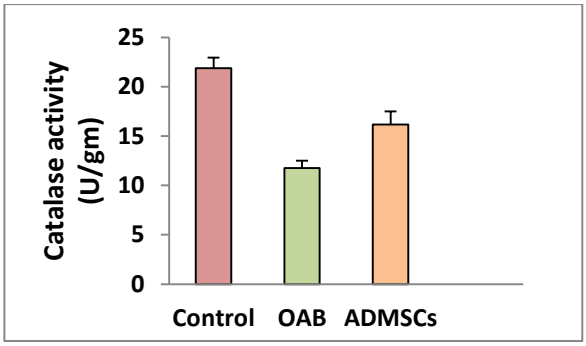

Figure (2): CAT levels of the studied groups.

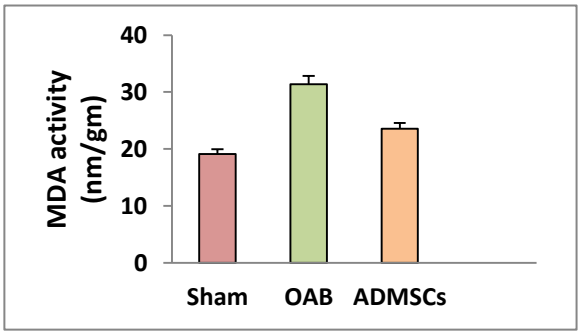

Figure (3): MDA levels of the studied groups.

2.9.Gene expression:

In the OAB group showed a highly significant ($p<0.001$) down regulation in BCL2 gene expression compared to control group. BCL2 gene expression was found to be markedly up regulated ($P<0.001$) in ADMSCs-treated group when compared with that of

obstructed rats group without treatment. Moreover, (Table 3, figure 4).

Table (3): BCL2 levels of the studied groups

Group / Variable	Control	OAB	ADMSCs
BCL2	0.986 ±0.0848	0.073± 0.0015[a]	0.532±0.056[ab]

Data are expressed as mean ± SE.

Significant difference compared to corresponding [a]control, and [b]OAB group by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons (Scheffé test) at $p \leq 0.05$.

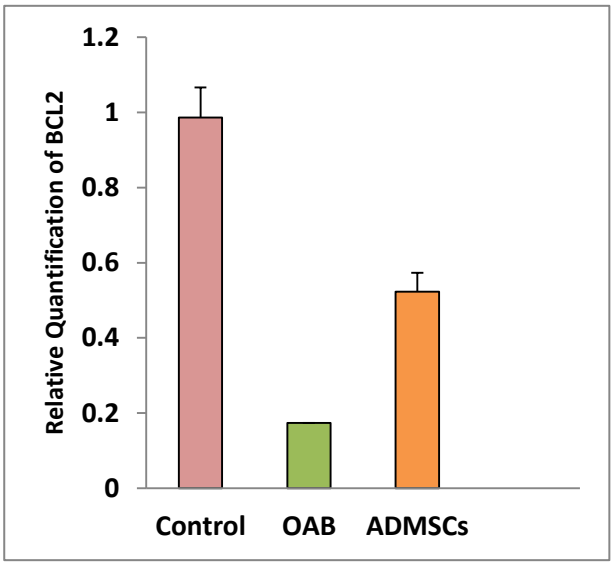

Figure (4): Gene expression of BCL2 for studied groups.

Impact of ADMSCs on bladder structure:

The bladder tissue of the control group showed normal structure of the bladder mucosa with intact urothelium with no sub -mucosal edema or inflammatory cell infiltration in the control group. In contrast to the control group, bladder sections from OAB group revealed sub-mucosal edema, congestion, fibrosis and neutrophils and many mononuclear cells infiltration (Figure 5) . On the other hand, the OAB treated with ADMSCs group showed sub-mucosal edema, congestion, and few neutrophils and mononuclear sells infiltration (Figure 5).

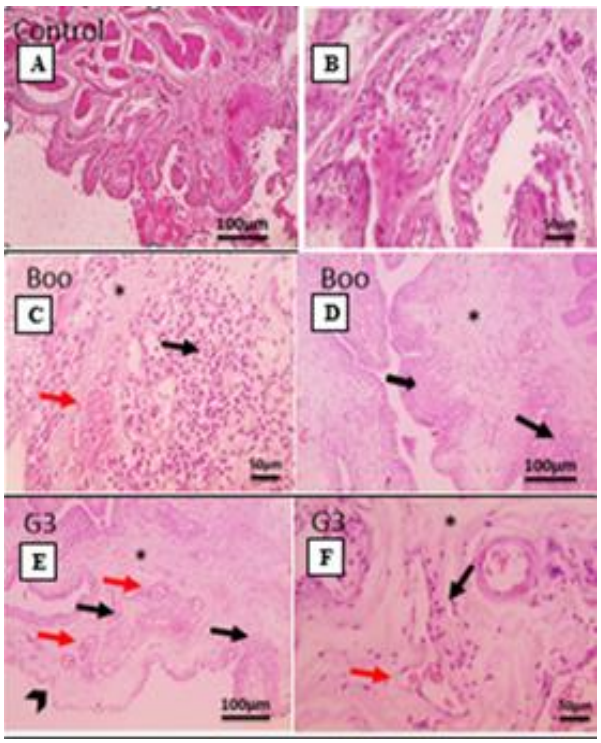

Figure (5): Microscopic investigation of H&E stained bladder tissue, exhibited; (A-F): Microscopic Photomicrograph of HE stained sections from bladder mucosa of adult rats in the control (A,B) and different experimental groups OAB (C&D), ADMSCs(E&F). X: 400. Sub-mucosal edema (*), congestion (red arrows), fibrosis (thick black arrows) and neutrophils and many mononuclear cells infiltration (thin black arrows).

Cell-based therapeutics have the power to completely change how diseases with high prevalence and associated social and economic costs are currently treated. For their therapeutic and reparative potential, mesenchymal stem cells (MSC) have been extensively studied in vivo, in vitro, and in clinical trials. In particular, the therapeutic effects of MSCs in the treatment of several bladder dysfunction disorders ,including OAB and detrusor under activity ,have been proven in preclinical studies[13]. In the current study we provide a promising approach for improving MSC state in the repair of BOO induced overactive bladder.

OAB induced by BOO is associated with profound smooth muscle cell (SMC) hypertrophy, bladder ischemia, and neurological injury, significant inflammation leading to bladder wall fibrosis and denervation super sensitivity [14]. Several mediators and

pathways are induced in response to BOO, including TGFβ, RhoA, HIF1α, and GRP78 [15].The intrinsic mechanism of apoptosis is facilitated by increased oxidative stress and mitochondrial malfunction brought on by OAB. This is achieved by the down regulation of BCL2, which results in a ratio of Bax to BCL2[16].

It has been found that oxidative stress is reflected by a variety of biomarkers, the majority of which indicate damage and oxidation of proteins, lipids, and DNA/RNA. Due to lipid peroxidation, malondialdehyde (MDA) is regarded as a helpful oxidative stress measure [17].

Different antioxidant defence systems guard against detrimental actions brought on by oxidative stress. These defence mechanisms comprise scavengers of free radicals that neutralize excessive ROS. Enzymes including glutathione peroxidase (GSH), catalase (CAT), and superoxide dismutase (SOD) are important components of these antioxidant systems. It has been observed that PBOO increases ROS production, which raises MDA and lowers CAT. Furthermore, under pathological circumstances, the collaboration of these antioxidants is critical for defence against oxidative stress. For instance, SOD changes reactive oxygen into hydrogen peroxide, which can also produce reactive radicals. Hydrogen peroxide is then further broken down into water and oxygen by CAT and GPX[18, 19].

Rats with BOO exhibit increased levels of MDA through our results. The current study further found that ADMSC therapy increased CAT activity and decreased MDA, demonstrating ADMSCs' capacity to both enhance antioxidant enzyme activity and shield cell membrane integrity from oxidative stress damage.

We explored the effect of ADMSCs to lessened bladder over activity showing significant increase compared to OAB rats.

According to the histological examination in the current study, the histological structure of OAB groups showed a significant alteration showing sub-mucosal edema, congestion, fibrosis, and neutrophils and many mononuclear cells infiltration while the treatment with ADMSCs showed an improvement with lower edema, congestion, and few neutrophils and mononuclear sells infiltration. In this regard, [20] examined the bladder tissues in OAB rats and reported that there was a significant alteration in the bladder tissues including urothelium is thinner, and the muscle bundles are separated with condensation of collagen.

In [21] study, Vacuolization of the urothelium was seen in H&E-stained bladder sections from the OAB group. There was also a noticeable widening of the intercellular gaps.

The increased permeability of epithelial cells to sodium ions, along with the synthesis of ATP and other mediators, may account for the mucosal damage observed in the blocked bladders. Additionally, these factors may contribute to the increased bladder afferent activity that results from outlet obstruction.

[22].

Chronic ischemia may be the cause of the changes in bladder structure and function that accompany OAB. In the same context, it was demonstrated that prolonged OAB in an animal model leads to decreased detrusor blood flow and oxygen tension, which is exacerbated by increases in detrusor pressure during filling.[23]. Additionally, fibroblasts and blood vessels may experience mitochondrial damage as a result of obstruction-mediated bladder wall ischemia. Similar to how a hypertrophic left ventricle brought on by hypertension may experience diastolic dysfunction, detrusor hypertrophy and fibrosis brought on by obstruction may eventually result in decreased contractility and ineffective bladder emptying, which is characterized by voiding dysfunction.[24].

The current study suggests that the ameliorative effect of ADMSCs against OAB may be due to both anti-oxidant, and anti-apoptotic properties of them.

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