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Original Research

Bladder dysfunction in hypoestrogenic rats with metabolic syndrome can be ameliorated after amniotic fluid stem cell treatment

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Abstract

Background: Bladder dysfunction may occur with high frequency in postmenopausal women with metabolic syndrome (MetS). This study evaluated the therapeutic effects of human amniotic fluid stem cells (hAFSCs) on bladder dysfunction in ovariectomized rats with MetS.

Materials and Methods: Forty-eight female rats were divided into 4 groups: normal control, ovariectomy (OVX), and OVX and MetS without (OVX + MetS) and with hAFSCs treatment (OVX + MetS + hAFSCs). We assessed cystometric parameters, serum biochemistry parameters, wall thickness of iliac artery, apoptotic cells and collagen volume in bladder tissues, and the expressions of purinergic and muscarinic receptors, apoptosis-associated mitochondrial proteins, and markers of inflammation, fibrosis, and oxidative stress at posttreatment 1 and 3 months.

Results: OVX + MetS rats showed significant dysfunction of bladder storage, including reduced intercontraction intervals and bladder capacity, along with increased residual urine volume and nonvoiding contractions. There was a significant increase in iliac artery wall thickness, bladder collagen volume, and number of apoptotic cells. Also, there were elevated expressions of P2X3 purinergic and M2/M3 muscarinic receptors, pro-apoptotic proteins, and markers of inflammation, fibrosis, and oxidative stress, with a concurrent decrease in anti-apoptotic protein, Bcl-2. Treatment with hAFSCs helped restoring bladder function, ameliorating histological abnormalities, and reducing pathological markers at 1 and/or 3 months.

Conclusion: These findings suggest that hAFSCs can effectively mitigate bladder dysfunction in rats with ovarian hormone deficiency and MetS by modulating oxidative stress and mitochondrial apoptotic pathways.

Key words: estrogen; menopause; metabolic syndrome; mitochondria; overactive bladder; stem cell.

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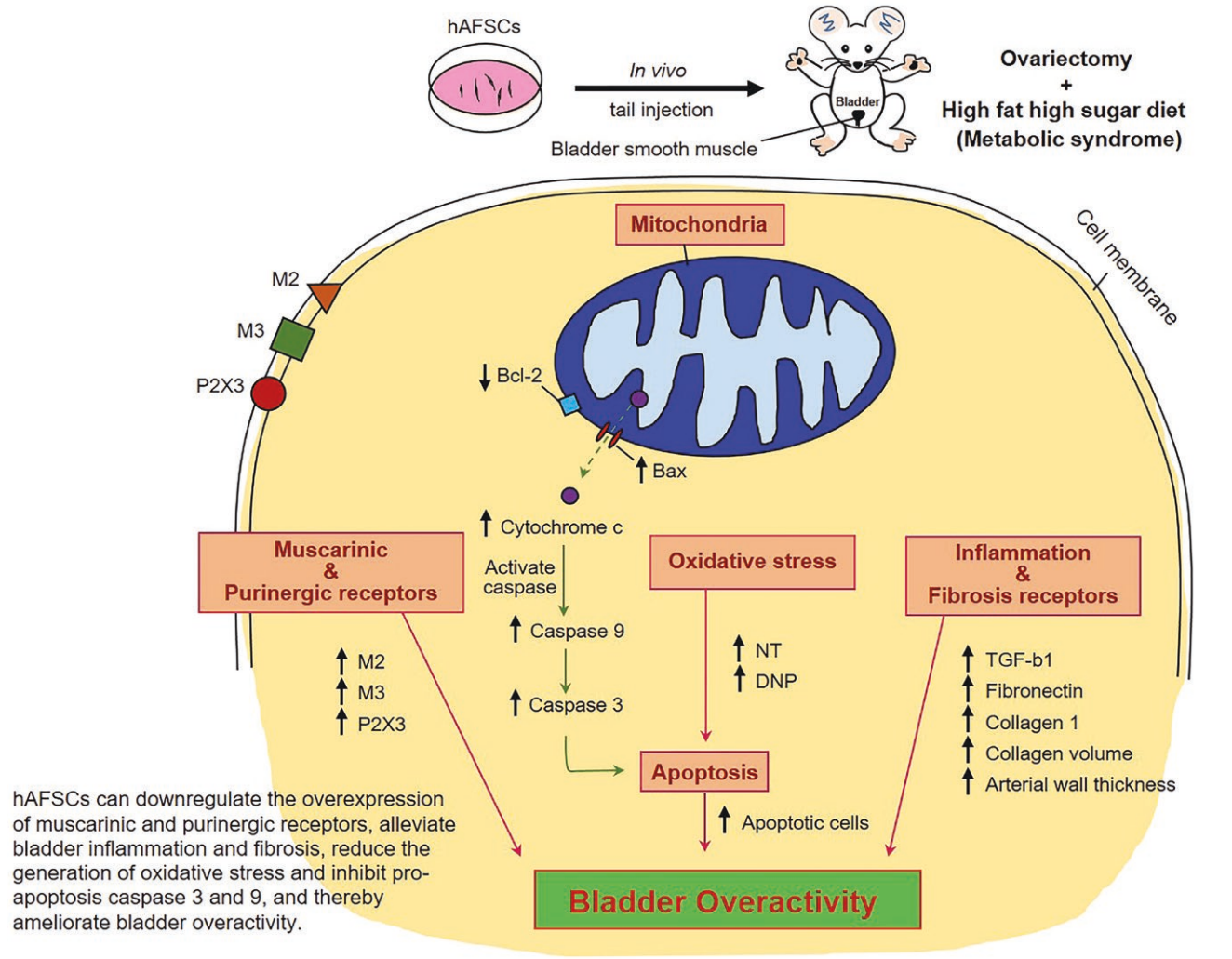
Graphical abstract



Significance Statement

Overactive bladder is a common condition in postmenopause women with metabolic syndrome (MetS); however, optimal and effective treatment for bladder dysfunction is still lacking. Our study demonstrates that human amniotic fluid stem cells (hAFSCs) can alleviate bladder inflammation and fibrosis, reduce the generation of oxidative stress, inhibit pro-apoptotic caspases, and thereby reduce bladder overactivity in ovariectomized rats with MetS. The results support the potential value of hAFSCs-based treatment of bladder dysfunction in postmenopausal women with MetS.

Introduction

Metabolic syndrome (MetS) is a multifactorial disorder characterized by a cluster of interrelated conditions, including abdominal obesity, hyperglycemia, hypertension, and dyslipidemia. Women with MetS have an elevated risk of developing cardiovascular disease, diabetes, and cancer,[1] particularly in the postmenopausal period.[2-5] The prevalence of MetS in postmenopausal women ranges from 13.7% to 55%,[3,5-7] with hypoestrogenism being a critical risk factor.[2,5,8] Clinical studies have demonstrated that overactive bladder is prevalent among women with MetS[9-11] and is more common in postmenopausal women.[12,13] Estrogen deficiency after menopause can lead to atrophic changes in urethral and vaginal tissues,[14] leading to a high prevalence of lower urinary tract symptoms, including frequent urination, urgency, and urinary incontinence.[12-14]

However, the underlining pathophysiology of MetS-induced bladder dysfunction is not clearly elucidated.[15] In vivo studies have demonstrated that hyperglycemia and MetS may induce oxidative stress, mitochondrial dysfunction, and apoptosis within bladder tissues. These mechanisms may lead to detrusor overactivity, commonly associated with diabetic neuropathy and myopathy.[16-18] Mitochondrial dysfunction in MetS is linked to an increase of oxidative stress[19] and apoptosis through increased reactive oxygen species (ROS) production that alters cellular redox potential.[20] Changes in mitochondrial function could contribute to bladder dysfunction in mice fed with long-term fructose diet.[21] Furthermore, upregulation

of M2/M3 muscarinic and purinergic receptors leads to detrusor overactivity in fructose-fed rats with MetS.[17,21,22]

There is no effective treatment for bladder dysfunction associated with MetS. Previous animal studies have demonstrated that treatment with human amniotic fluid stem cells (hAFSCs) can improve bladder dysfunction caused by diabetes.[23] Additionally, several preclinical experiments have employed stem cells to address various bladder dysfunctions.[24] However, the role of stem cells in treating MetS-induced bladder dysfunction remains to be explored. This study aims to investigate the effects of hAFSCs on the recovery of bladder dysfunction in ovariectomized rats with MetS.

Methods

Experiment design

All animal care and experimental protocols were conducted in accordance with the guidelines established by the Institutional Ethics Committee for the Care and Use of Experimental Animals (Approval no. 2020121901) and the Institutional Review Board of our institute (Approval no. 202002270A3). The quality of animal experiments was evaluated using the ARRIVE guidelines.

Forty-eight female Sprague-Dawley rats (10-12 weeks old) were allocated into 4 groups: Group 1, normal control (control); Group 2, bilateral ovariectomy (OVX); Group 3, OVX and MetS (OVX + MetS); and Group 4, OVX and MetS treated with single injection of 3 × 1 000 000 hAFSCs via tail vein (OVX + MetS + hAFSCs). Ovariectomized rats were fed with a high fat and high sugar (HFHS) diet for 6 months to induce MetS (OVX + MetS). The treatment of hAFSCs was given in OVX + MetS + hAFSCs group. Conscious cystometry were performed in all rats at 1 and 3 months following hAFSCs injection ($n = 6$ per time point in each group). Blood samples were collected before conscious cystometry at 1 and 3 m for biochemical analysis and measurement of estrogenic hormonal levels. The common iliac artery wall thickness, bladder apoptotic cells, and collagen volume were evaluated using hematoxylin and eosin, Masson's trichrome, and apoptotic cell staining. The levels of immunoreactivity and mRNA expression of muscarinic and purinergic receptors, bladder inflammation and fibrosis markers, apoptosis-associated mitochondrial proteins, and oxidative stress markers were also assessed by immunohistochemistry/immunofluorescence staining and reverse transcription polymerase chain reaction. The experimental workflow of this study is shown in Supplementary Figure S1.

Animal models of OVX and MetS

In OVX rats, surgical ovariectomy was performed by removing bilateral ovaries through abdominal incisions under isoflurane anesthesia to mimic the postmenopausal condition, with efforts made to reduce suffering and the number of animals. In the OVX + MetS group, rats underwent OVX, followed by HFHS diet 2 weeks later and continued for the following 6 months. The HFHS diet (D19091601, Research Diets Inc., New Brunswick, NJ, USA) consisted primarily of casein, soybean oil, and fructose, as previously reported.[25] Rats in the normal control group were fed with a regular standard diet (LabDiet 5001, LabDiet, St. Louis, MO, USA).

Isolation and culture of hAFSCs for treatment

HAFSCs were derived from fresh amniotic fluid obtained from healthy pregnant donors through routine amniocentesis after informed consent. Cells were cultured in StemPro MSC serum-free medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C with 5% carbon dioxide. Flow cytometry was used to characterize the specific surface antigens of hAFSCs, as described in our previous study.[23] The hAFSCs at passage 3-6 were collected and prepared to a final concentration of 3 × 1 000 000 cells in 0.3-mL phosphate-buffered saline (PBS). Under inhalation anesthesia, 3 × 1 000 000 hAFSCs were administered via tail vein injection in the hAFSCs-treatment group.

Physical indicators and biochemical parameters

During the experiment, physical parameters such as waist circumference, body weight, systolic pressure, diastolic pressure, and mean arterial pressure were measured monthly. All rats were placed in individual metabolic cages to facilitate the measurement of 24-hour water intake and urine output. Blood samples were collected from the femoral vein for the analysis of estrogen hormone levels using a 17-β estradiol enzyme-linked immunosorbent assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). At the time of euthanasia, blood samples were collected from the heart of rats to obtain biochemical data. The biochemical parameters assessed included fasting blood glucose, glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), triglycerides, low-density lipoprotein (LDL), total cholesterol, high-density lipoprotein (HDL), and lactate dehydrogenase (LDH). These measurements were performed using an automated analyzer (Hitachi 7150, Tokyo, Japan) according to the manufacturer's instructions.[26]

Conscious cystometry

Two days after the implantation of a suprapubic catheter, the animals were placed in metabolic cages (Med Associates Inc., St. Albans, VT, USA) for conscious cystometry at 1 and 3 months post-hAFSCs treatment, following the protocol of our prior study.[27] Cystometric parameters, including peak voided volume, peak voiding pressure, intercontraction interval, bladder capacity, nonvoiding contraction, and residual urine volume, were analyzed over 5 consecutive micturition cycles. Cystometric analysis was performed using Cystometry Analysis Version 1.05 (Catamount Research and Development, St. Albans, VT).

Histological studies

Following cystometry, the common iliac arteries and bladders were collected for histological and microbiological examinations.

For histological examination, segments of the common iliac arteries were initially fixed in 4% paraformaldehyde for 2.5 hours at 4 °C. Subsequently, the specimens were transferred to 25% sucrose in PBS at 4 °C until the blocks sank. The specimens were then fixed in optimal cutting temperature compound and sectioned into 10-μm slices. These sections were stained with hematoxylin and eosin. The thickness of the common iliac arterial wall thickness was quantified by averaging measurements taken at 4 distinct locations per sample.[27]

The dissected bladders were fixed in optimal cutting temperature compound, frozen in dry ice powder, and stored at −70 °C. Cryosections were prepared at a thickness of 10-μm thickness at −18 °C and subsequently stained with Masson's trichrome stain Kit (DAKO, Glostrup, Denmark).

The percentage of fibrosis formation in bladder tissue was quantified by calculating the ratio of collagen area to smooth muscle area, as outlined in our prior study.[28] Digital images of each section were captured in 10 random, nonoverlapping fields at 400× magnification. These images were then analyzed and compared among the experimental groups. The volumes for collagen and total bladder tissue were quantified using Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA).

For the detection of apoptotic cell, bladder tissue sections were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an in-situ cell death detection kit (Roche, Pleasanton, CA, USA). The number of TUNEL-positive cells was quantified in 10 randomly selected nonoverlapping fields of view at 400× magnification. These counts were then compared among experimental groups, as detailed in our previous study.[29]

Immunohistochemistry and immunofluorescence assessment

Bladder sections were immunostained to detect muscarinic receptors (M2 and M3), purinergic receptor (P2X3), inflammation and fibrosis markers (transforming growth factor beta 1 [TGF-β1], fibronectin and collagen 1, as well as apoptosis-associated mitochondrial proteins [Bcl-2 associated X (Bax), B-cell lymphoma-2 (Bcl-2), caspase-3, caspase-9, and cytochrome c utilizing the avidin-biotin-peroxidase complex (ABC) method (PK-6101, Vector Laboratories, Burlingame, CA, USA)]). Fresh-frozen sections were initially fixed in ice-cold acetone for 10 minutes and then air-dried. After blocking with 2% normal goat serum for 1 hour, the sections were incubated overnight at 4 °C with a primary antibody targeting the receptors and markers. Endogenous peroxidase activity was quenched by incubating the sections with 0.1% H_2O_2 and 100 % methanol for 30 minutes. Following PBS washing, the sections were incubated with biotinylated secondary antibodies for 1 hour, followed by a 60-minute incubation with the avidin-biotin-horseradish peroxidase complex. Staining was visualized using 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA). The catalogue numbers, dilution concentrations, host species, isotypes, and manufacturer of each antibody are summarized in Supplementary Table S1.

Immunofluorescence was employed to analyze the expressions of oxidative stress markers, nitrotyrosine (NT) and 2,4-dinitrophenol (DNP), in bladder sections. The sections were treated with 4% paraformaldehyde for 5 minutes and subsequently washed 3 times with PBS. After incubating with 1% bovine serum albumin blocking reagent and 0.1% Triton-X 100, cells underwent PBS washing and were then exposed to primary antibodies at 4 °C for 20 hours. The primary antibodies were then detected after incubating the sections with secondary antibody Alexa-flor 488 (1:250, Invitrogen, Grand Island, NY, USA) for 1 hour. Nuclear staining was conducted using 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence analysis was performed using an Olympus BX-51 microscope equipped with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Negative controls were prepared using the same tissue blocks, omitting the specific primary antibodies.

Immunoreactivity analysis was conducted using Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA) under Olympus BX-51 microscope. The ratio of immunoreactivity levels in OVX + MetS rats with or without hAFSCs treatment relative to control rats was calculated.

Real-time PCR of bladder

Real-time PCR was performed following the manufacturer's protocol (Life Technologies, Grand Island, NY). Total RNAs were extracted using a Trizol reagent (Invitrogen) and reverse transcribed at 25 °C for 5 minutes, 50 °C for 1 hour, and 70 °C for 15 minutes, followed by cooling to 4 °C for 5 minutes. Gene expressions for M2, M3, P2X3, TGF-β1, fibronectin, collagen 1, Bax, Bcl-2, caspase-3, caspase-9, and cytochrome c in bladder tissue were conducted using inventoried TaqMan assays from Applied Biosystems (Life Technologies). Specific assay codes were M2 (Rn02532311-s1), M3 (Rn00560986-s1), P2X3 (Rn00579301-m1), TGF-β1 (Rn00572010-m1), fibronectin (Rn00569575-m1), collagen 1 (Rn01463848-m1), Bcl-2 (Rn99999125-m1), Bax (Rn01480161-g1), cytochrome c (Rn00470541-g1), caspase-3 (Rn00563902-m1), and caspase-9 (Rn00581212-m1) obtained from Applied Biosystems (Oster City, CA, USA). GAPDH (Rs99999916-s1) was served as an endogenous control for quantification of relative gene expression. Thermal cycling and fluorescence detection were carried out on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR conditions included an initial step at 50 °C for 2 minutes, followed by 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. Data were analyzed using the 2[-Delta Delta C(T)] method.[30] The mRNA expression ratios of OVX + MetS rats with or without hAFSCs treatment relative to control rats were determined. Values were aggregated and expressed as mean ± SD and compared statistically across groups and time points within each group.

Statistical analysis

Data analysis was conducted utilizing Prism 5 (GraphPad Software, Inc.). Continuous variables were expressed as mean ± SD. One-way analysis of variation was employed to compare continuous data across different groups, followed by post hoc analysis using Tukey–Kramer test for multiple comparisons. The impact of hAFSCs within each group was evaluated using chi-square tests with Fisher's exact test. A *P*-value of <.05 was considered statistical significance.

Results

Physical indicators and biochemical parameters

Compared with the normal control and OVX groups, OVX + MetS rats exhibited significantly increased systolic blood pressure, body weight, fasting blood glucose, triglycerides, cholesterol, GOT, GPT, LDL, and LDH levels, while HDL levels, 24-hour urine output, 24-hour water intake, and bladder weight were significantly decreased (P <.05, Supplementary Table S2). Although most physical indicators and biochemical parameters in OVX + MetS rats showed improvement at 1 and/or 3 months following hAFSCs treatment, only the reduction in GOT, GPT, and LDL levels reached statistical significance (P <.05).

Cystometric results and muscarinic/purinergic receptor expression

Compared with normal control group, OVX group exhibited a significant reduction in peak voided volume, intercontraction interval, and bladder capacity at 1 and/or 3 months (P <.05).

The OVX + MetS group also demonstrated significant impairment of the 5 parameters except peak voiding pressure at 1 and 3 m compared with normal controls. Also, there was significantly lower intercontraction interval and bladder capacity, coupled with an increased number of nonvoiding contractions at 1 and/or 3 months compared with OVX group. Treatment of hAFSCs (OVX + MetS + hAFSCs group) led to the recovery of residual volume and nonvoiding contraction to normal control levels at 1 and 3 months but no significance compared with OVX group. However, there was a significant improvement of peak voided volume, intercontraction interval, bladder capacity, residual urine volume, and nonvoiding contraction at 1 month (P <.05) but no significance at 3 months compared with OVX + MetS group (Figure 1).

Compared with normal control group and/or OVX group, OVX + MetS group exhibited significantly elevated immunoreactivities and mRNA expressions of M2, M3, and P2X3 at 1 and/or 3 months (P <.05). However, these parameters returned to normal control levels at 1 and/or 3 months following hAFSCs treatment (immunostaining in Figure 2A and E, and mRNA in Figure 3A).

Inflammation and fibrosis markers expression and iliac arterial wall thickness

Compared with normal control and OVX groups, OVX + MetS rats exhibited significantly increased immunoreactivities and mRNA expressions of inflammation and fibrosis markers, including TGF-β1, fibronectin, and collagen 1 at 1 and/or 3 months (P <.05). However, these markers showed a significant improvement and returned to normal control levels at 1 and/or 3 months post hAFSCs treatment (P <.05, immunostaining in Figure 2B and E, and mRNA in Figure 3B).

The OVX + MetS rats had significantly increased common iliac arterial wall thickness and bladder collagen volume compared with normal control group (P <.05). Both parameters showed a significant improvement and recovered to normal control levels at 1 and 3 months following hAFSCs treatment (P <.05, Figures 4 and 5).

Oxidative stress markers expression

The OVX + MetS group exhibited significantly increased immunoreactivities of oxidative stress markers, such as NT and DNP, compared with the normal control and OVX groups (P <.05). These levels were significantly improved and recovered to normal control levels at 1 and/or 3 months following hAFSCs treatment (P <.05, Figure 2C and E).

Apoptotic cells and apoptosis-associated mitochondrial proteins

The OVX + MetS group demonstrated a significant increase in apoptotic cells within the bladder tissue compared with the normal control and OVX groups (P <.05). This increase was significantly reduced and returned to normal control level at 1 and 3 months post-hAFSCs treatment (P <.05, Figure 6).

Compared with normal control and OVX groups, OVX + MetS group exhibited significantly reduced immunoreactivity and mRNA expression of Bcl-2 (P <.05). However, OVX + MetS group showed a significant increase in the immunoreactivities of Bax, caspase-3, caspase-9, and cytochrome c at 1 and 3 months and increased mRNA expressions of caspase-3 and caspase-9 at 1 month (P <.05). Following hAFSCs treatment, the immunoreactivity and mRNA expression of caspase-3 and caspase-9 levels showed significant improvement at 1 month. Also, the immunoreactivity and mRNA expression of Bcl-2 showed significant improvement at 1 month and 1 and 3 months, respectively (P <.05, immunostaining in Figure 2D and E, and mRNA in Figure 3C).

Discussion

The current study demonstrates that ovariectomized rats, when subjected to HFHS diet to develop MetS, have increased waist circumference, hyperglycemia, hypertension, and dyslipidemia. Previous studies have demonstrated that rats with MetS exhibit increased micturition frequency and peak voiding pressure, along with decreased intercontraction interval and bladder capacity compared with normal rats.[25] Ovariectomized rats with MetS also display exacerbated micturition frequency and nonvoiding contractions, coupled with decreased voided volume.[25] In our study, OVX + MetS rats exhibited bladder storage dysfunction, including short-intercontraction interval, low peak voided volume, high residual urine volume, low bladder capacity, and increased nonvoiding contractions, consistent with bladder overactivity. A prospective study highlighted a correlation between MetS and overactive bladder in women experiencing lower urinary tract symptoms.[11] Among women with MetS, 84% were diagnosed symptomatically with overactive bladder and 36% were found to have detrusor overactivity through urodynamics. The overactive bladder group had significantly higher waist circumference, lower HDL levels and higher blood pressure, compared with the nonoveractive bladder group.[10] In our study, although hAFSCs treatment improved only biochemical parameters such as GOT, GPT, and LDL levels in OVX + MetS rats, significant improvement in bladder storage dysfunction was observed. Prior studies have demonstrated that stem cells derived from cord blood and adipose tissue can improve cystometric results in diabetic animal models, albeit without significant impact on hyperglycemia.[31,32] Several animal studies have reported that stem cells can ameliorate diabetic bladder dysfunctions.[23,31,32] However, the precise therapeutic mechanisms of stem cells in treating MetS-related bladder dysfunction remain incompletely understood.

Previous study has suggested that MetS could contribute to the underlying mechanisms for developing overactive bladder syndrome, including autonomic nervous system dysfunction, chronic ischemia, chronic pro-inflammation, bladder mucosal insulin resistance, and dysbiosis, and advocated that MetS and overactive bladder syndrome may share common pathophysiology.[15] Mitochondrial function has also been reported to be associated with MetS-related oxidative stress.[19] Mitochondria in the smooth muscle layer of bladder were disrupted and swollen in rats with MetS induced by fructose feeding for 3 or 6 months.[17,21] HFHS feeding and ovarian hormone deficiency can enhance the generation of oxidative stress mediated through mitochondrial pathway.[25] Based on the aforementioned preclinical studies on MetS and bladder dysfunction, and our results in this study demonstrating bladder overactivity in OVX + MetS rats can be ameliorated following hAFSCs treatment, we speculate that hAFSCs may improve autonomic nervous system dysfunction, chronic ischemia, and pro-inflammation in rats through the mitochondrial pathway, thereby restoring bladder function.

Bladder function in MetS rats is intricately linked to alterations in the motor and sensory functions of muscarinic

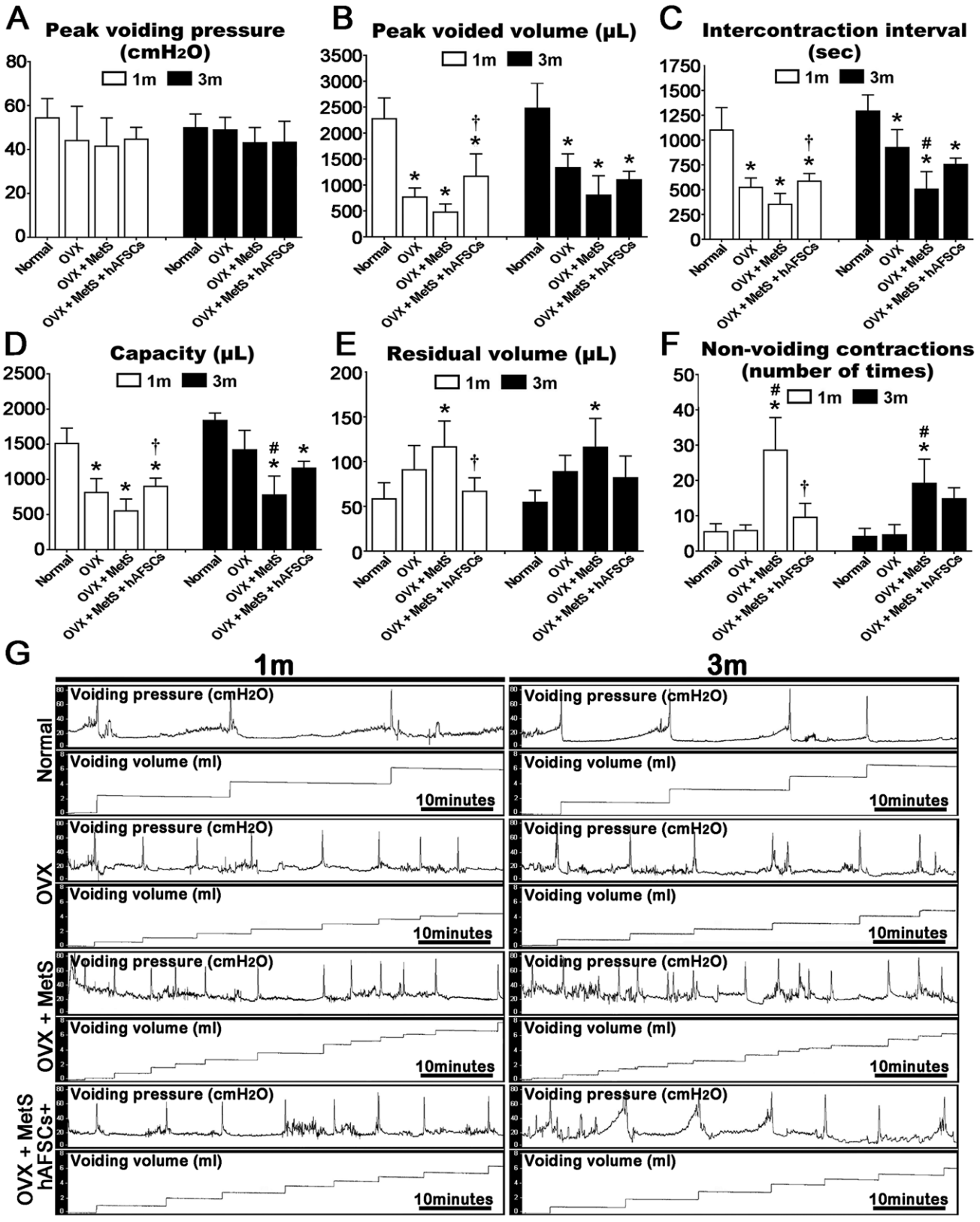


Figure 1. Cystometric results of all groups are presented. In upper panel, cystometric variables include (A) peak voiding pressure, (B) peak voided volume, (C) intercontraction interval, (D) bladder capacity, (E) residual volume, and (F) nonvoiding contraction. Lower panel (G) depicts peak voiding pressure and voided volume at 1 and 3 m after ovariectomy (OVX), OVX + metabolic syndrome (MetS), OVX + MetS + hAFSCs, and normal controls. Compared with normal controls, OVX causes impairment of peak voided volume, intercontraction interval, and capacity at 1 and/or 3 m. If OVX rats have MetS (OVX + MetS), there is significant impairment of the 5 parameters except peak voiding pressure at 1 and 3 m compared with normal controls and significantly deteriorated intercontraction interval, bladder capacity, and nonvoiding contraction at 1 and/or 3 m compared with OVX group. The treatment of hAFSCs causes recovery of residual volume and nonvoiding contraction to normal control levels at 1 and 3 m, no significance compared with OVX group, but significant improvement of peak voided volume, intercontraction interval, bladder capacity, residual urine volume, and nonvoiding contraction at 1 m but no significance at 3 m compared with OVX + MetS group. However, there is still impaired peak voided volume, intercontraction interval, and bladder capacity compared with normal control group. $^{*}P < .05$ vs normal control; $^{\#}P < .05$ vs OVX; $^{\dagger}P < .05$ vs OVX + MetS. $N = 6$ at each time point in each group. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m, 3 months.

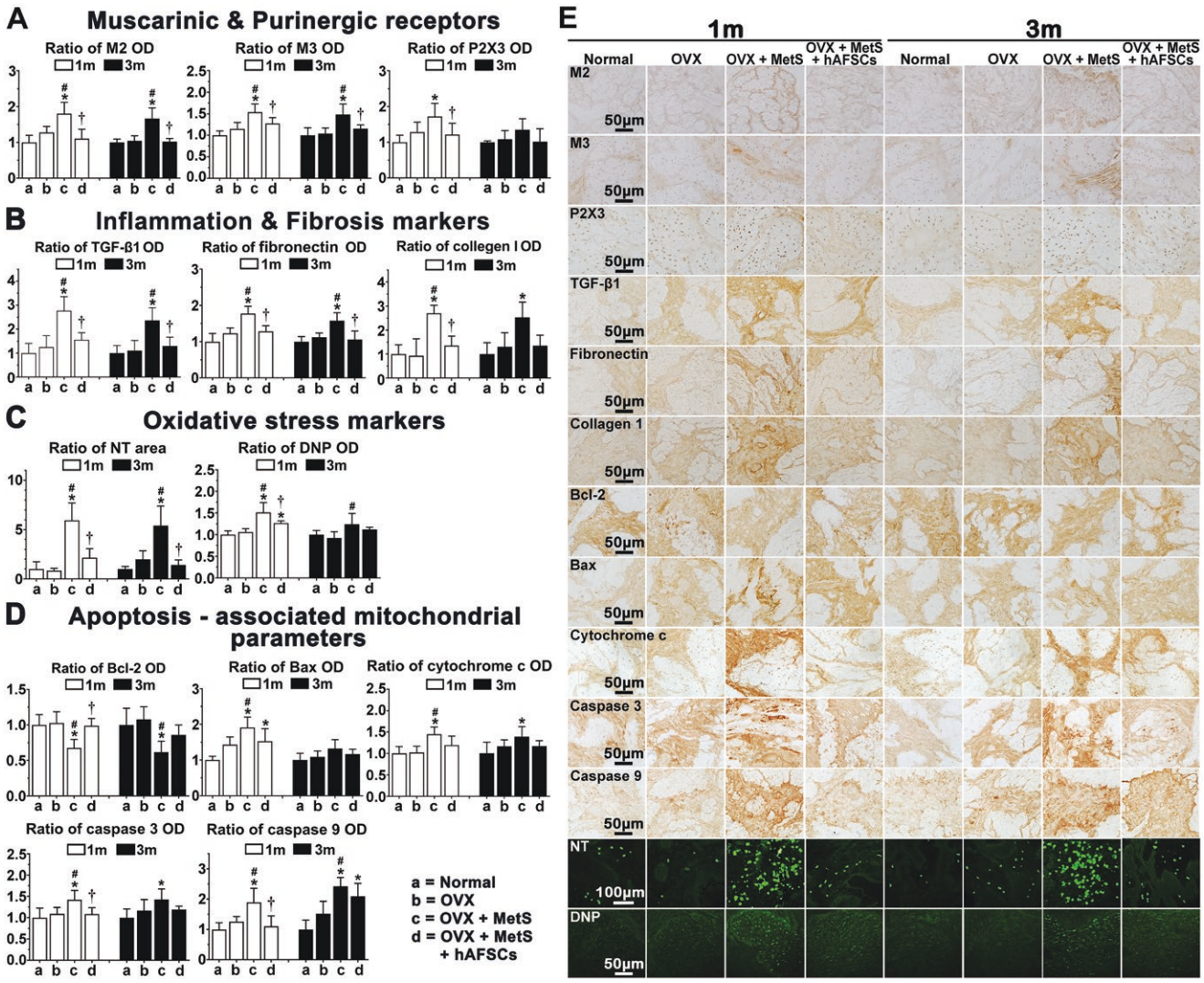


Figure 2. Expressions of the immunoreactivity of muscarinic/purinergic receptors (A), inflammation and fibrosis markers (B), oxidative stress markers (C), apoptosis-associated mitochondrial proteins (D), and temporal profiles of immunohistochemistry and immunofluorescence expressions (E). Compared with normal control group, ovariectomy (OVX) causes nonsignificant changes of all parameters at 1 and 3 m. Compared with normal control group and/or OVX group, OVX rats with metabolic syndrome (OVX + MetS) cause significantly increased immunoreactivities of M2, M3, P2X3, TGF-β1, fibronectin, collagen 1, NT, DNP, Bax, cytochrome c, caspase-3, and caspase-9 at 1 and/or 3 m but significantly reduced Bcl-2 immunoreactivity at 1 and 3 m. Compared with OVX + MetS group, hAFSCs treatment (OVX + MetS + hAFSCs) causes significant improvement of all the parameters except Bax and cytochrome c at 1 and/or 3 m and P2X3, collagen 1, DNP, Bcl-2, caspase-3, and caspase-9 at 3 m. Compared with normal controls, hAFSCs treatment (OVX + MetS + hAFSCs) causes recovery of all the parameters except DNP and Bax at 1 m and caspase-9 at 3 m. $^{*}P < .05$ vs normal control; $^{\#}P < .05$ vs OVX; $^{\dagger}P < .05$ vs OVX + MetS. $N = 6$ at each time point in each group. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m,3 months.

mechanism.[17,22,25,33] Previous studies have highlighted the upregulation of muscarinic M2 and M3 expressions along with dysregulation of smoothelin, contributing to symptoms of overactive bladder in MetS rats.[17,22] Enhanced protein expressions of M2, M3, and P2X3 receptors have been observed in female HFHS-fed MetS rats,[21,25,33] and surgical OVX further enhances these expressions.[25,33] In our study, compared with normal control and OVX groups, the OVX + MetS group exhibited significant increases in proteins and mRNA expressions of M2, M3, and P2X3 receptors. However, these expressions recovered to control levels at 1 and/or 3 months following hAFSCs treatment.

The pathophysiology of bladder dysfunction induced by MetS and ovarian hormone deficiency remains poorly understood. Preclinical animal studies have demonstrated that MetS rats fed with HFHS diet for 6 months develop bladder fibrosis and symptoms of overactive bladder,[25,33] while surgical OVX can aggravate bladder oxidative stress damage and apoptotic cells.[29] Our findings demonstrate that the OVX + MetS group exhibited significantly increased collagen volume and collagen/smooth muscle ratio, and elevated levels of inflammation and fibrosis markers such as TGF-β1, fibronectin, and collagen 1. Moreover, ovariectomized rats with MetS (OVX + MetS) showed thicker walls in the common iliac arteries compared with control and OVX groups, suggesting a potential link between bladder overactivity and atherosclerosis. In our study, we observed that the rats fed with a 2% cholesterol diet for 8 weeks and subjected to balloon endothelial injury in the common iliac artery developed artery atherosclerosis and ischemic bladders characterized by arterial

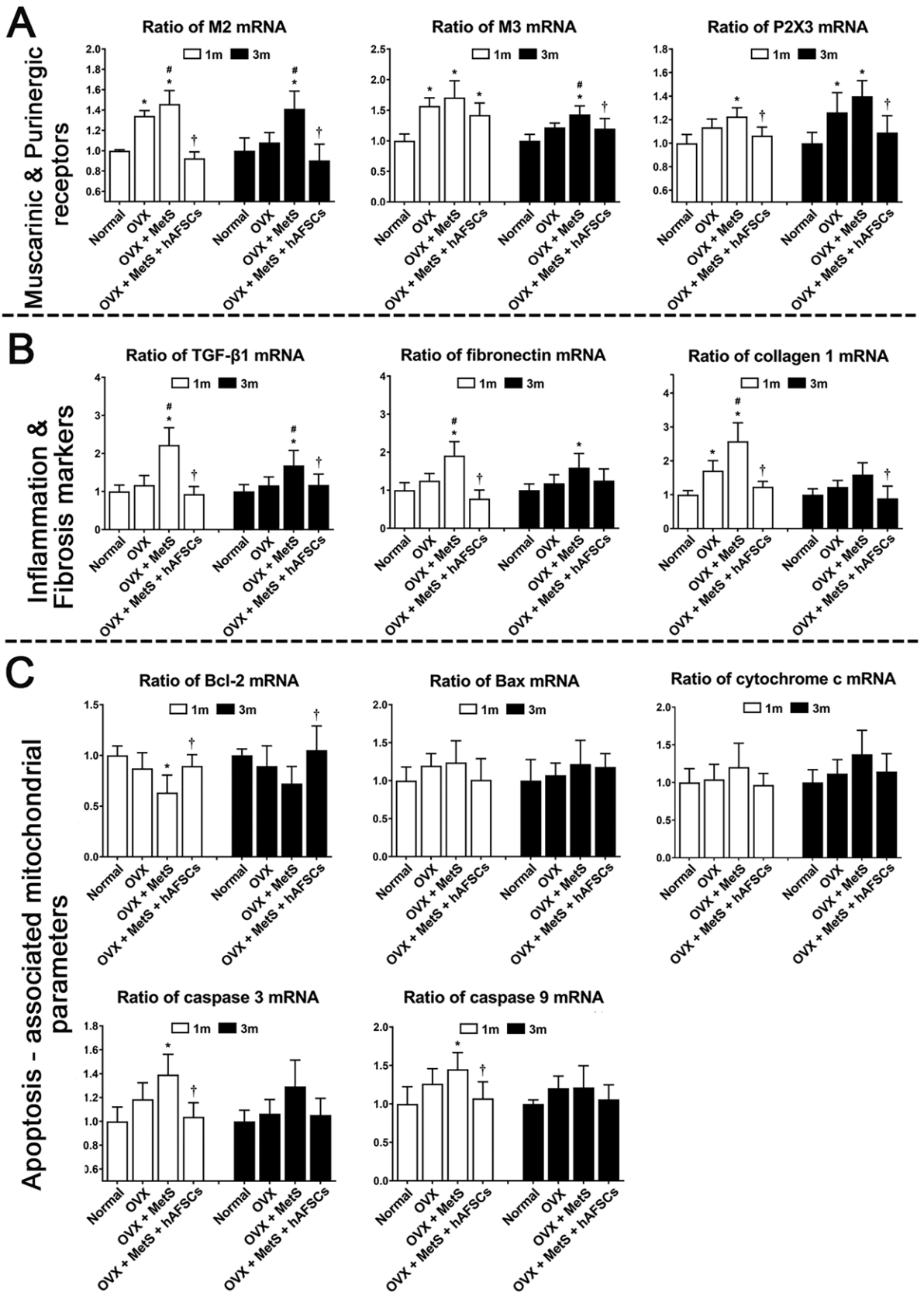


Figure 3. Relative mRNA expressions of muscarinic/purinergic receptors (A), inflammation and fibrosis markers (B), and apoptosis-associated mitochondrial proteins (C). Compared with normal control group, ovariectomy (OVX) causes significantly increased mRNA expressions of M2/M3 and collagen 1 at 1 m and P2X3 at 3 m. When compared with normal control and/or OVX groups, ovariectomized rats with MetS (OVX + MetS) causes significantly increased mRNA expressions of M2, M3, P2X3, TGF-β1, fibronectin, collagen 1, caspase-3, and caspase-9 at 1 and/or 3 m but significantly reduced Bcl-2 mRNA expression at 1 m. Compared with OVX + MetS group, hAFSCs treatment (OVX + MetS + hAFSCs) causes significant improvement of all the parameters at 1 and/or 3 m except no significant change of Bax and cytochrome c at 1 and 3 m. Compared with normal controls, hAFSCs treatment (OVX + MetS + hAFSCs) causes no significant difference of all the parameters except M3 at 1 m.$^{*}P < .05$ vs normal control; $^{\#}P < .05$ vs OVX; $^{\dagger}P < .05$ vs OVX + MetS. $N = 6$ at each time point in each group. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m, 3 months.

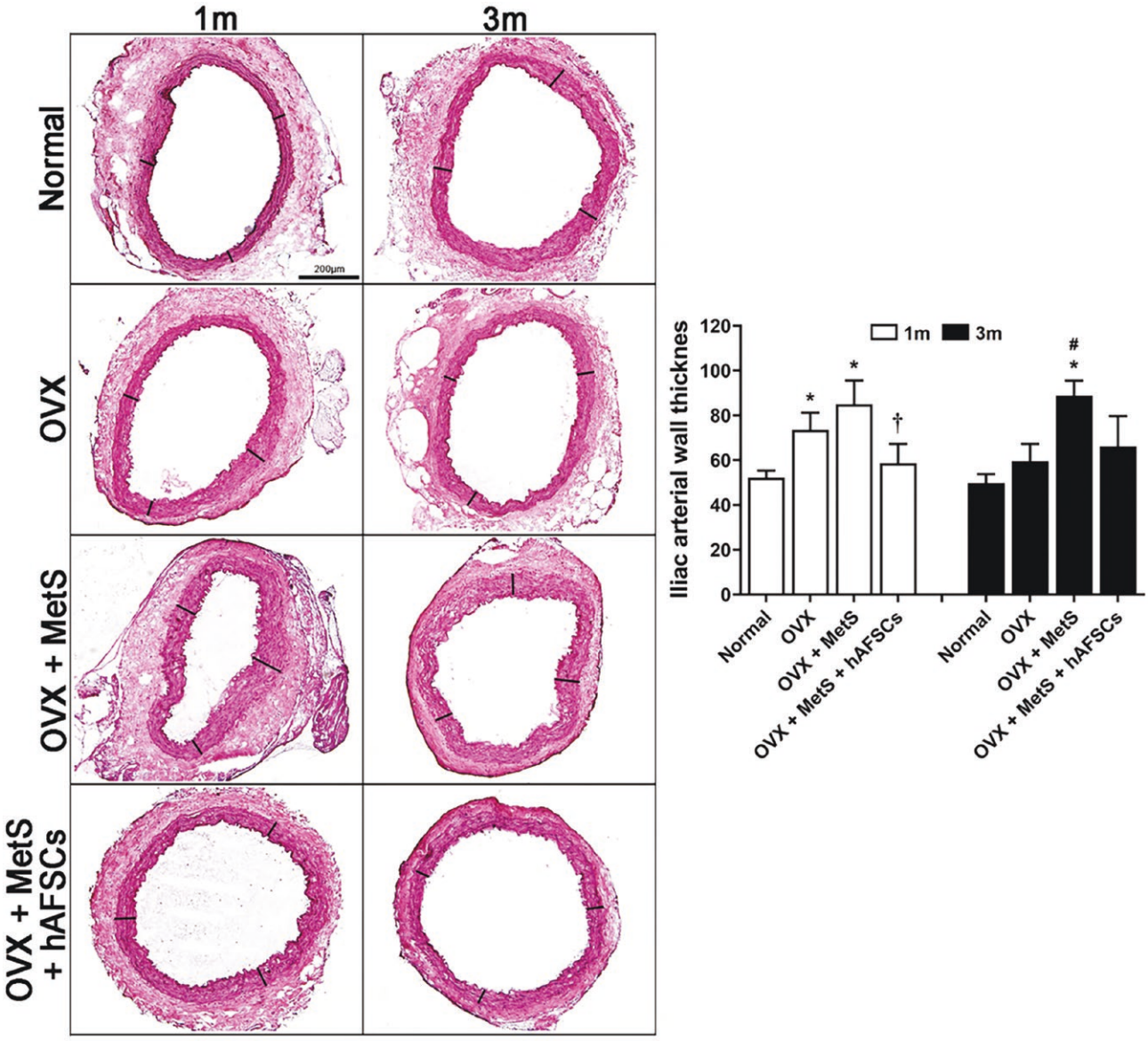


Figure 4. Hematoxylin and eosin staining of the common iliac artery. Compared with normal control group, OVX group has significantly increased wall thickness of the common iliac artery at 1 m. Ovariectomized rats with MetS (OVX + MetS) have significantly increased wall thickness at 1 and 3 m when compared with normal controls and at 3 m when compared with OVX group. After hAFSCs treatment, the wall thickness has significant improvement at 1 m when compared with OVX + MetS group and recovers to normal control level at 1 and 3 m. *Compared with normal control, $P < .05$; #Compared with OVX group, $P < .05$; †Compared with OVX + MetS group, $P < .05$. $N = 6$ at each time point in each group. Scale bar indicates 200 μm. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m, 3 months.

wall thickening and overactive bladder symptoms.[27] In the current study, hAFSCs treatment in OVX + MetS rats led to improvements in bladder collagen volume, common iliac arterial wall thickness, inflammation, and fibrosis marker expression, and contributed to the recovery of bladder function.

Our findings also revealed that OVX + MetS rats exhibited increment in the levels of bladder oxidative stress damage and apoptotic cells compared with normal controls and OVX rats. Oxidative stress can induce oxidative damage to bladder cells, leading to impaired mitochondrial function and cell apoptosis, which clinically manifests as bladder dysfunction. Previous animal studies have linked detrusor overactivity in rats with MetS induced by a 6-month fructose diet to the increased nitrotyrosine (NT) levels in bladder tissue.[17] The expression of oxidative stress markers such as DNP and NT was significantly higher in MetS and OVX + MetS rats fed with HFHS diet for 6 months compared with normal controls.[25] Hyperglycemia is also known to enhance ROS production,[19] highlighting the potential therapeutic benefit of ROS inhibition in treating MetS-related bladder dysfunction. Our study further shows that the immunoreactivities of oxidative stress markers, including NT and DNP, were significantly elevated in the OVX + MetS group but notably improved following hAFSCs treatment.

In addition to oxidative stress damage in bladder, several studies suggest that hyperglycemia and metabolic disorders can cause mitochondrial dysfunction and promote an increase of apoptosis in bladder tissues.[16,18] In a 6-month fructose-fed rat model of MetS, hyperglycemia contributes to the elevated ROS production, impairs mitochondrial ATP production, and

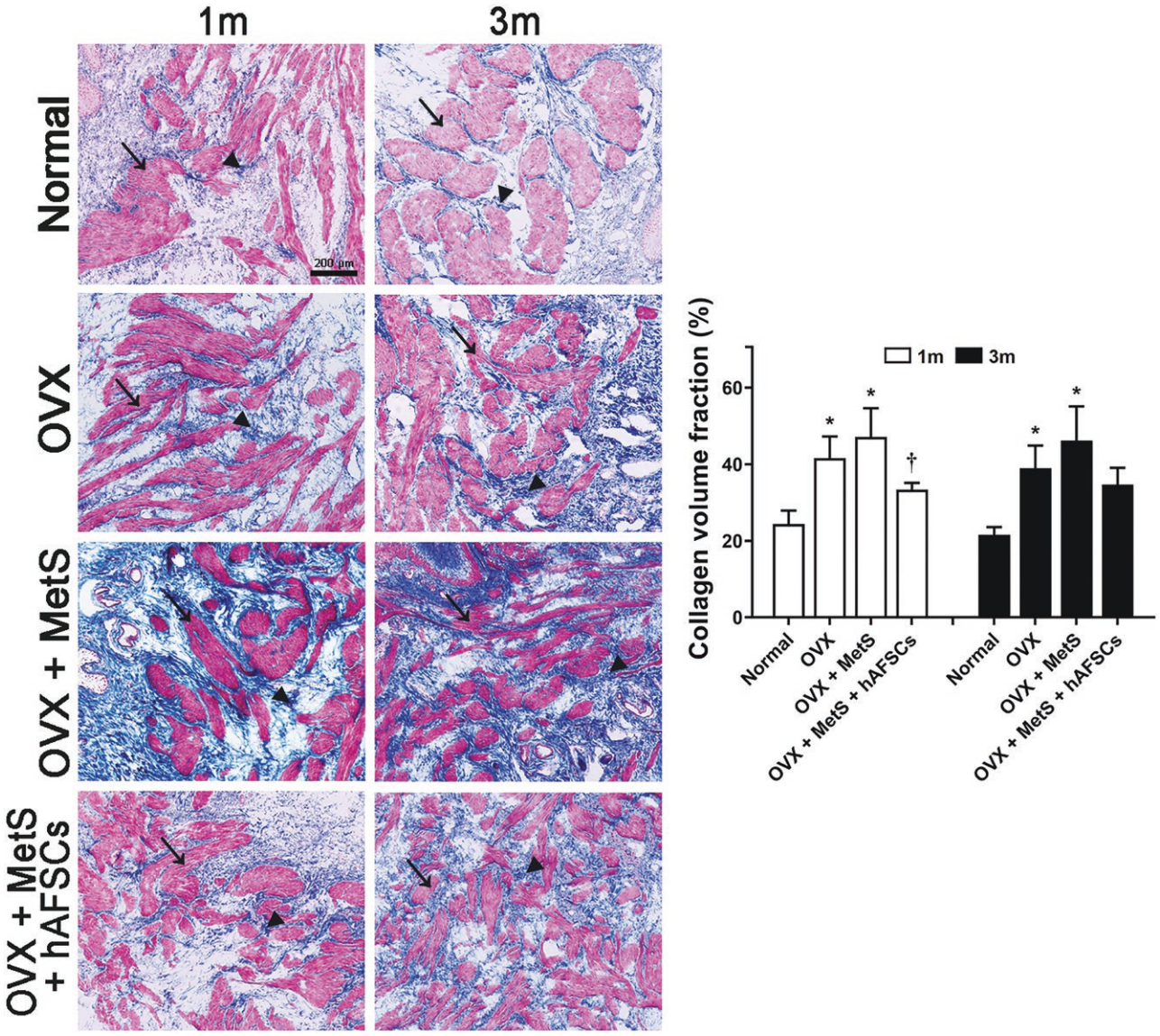


Figure 5. Histological features of bladder damage as represented by Masson's trichrome stain. The collagen is stained blue (arrowheads) and the muscle red (arrows). Compared with normal control, OVX and OVX + MetS groups have significantly increased bladder collagen volume at 1 and 3 m. After hAFSCs treatment, bladder collagen volume is significantly improved when compared with OVX + MetS group at 1 m and recovers to normal control level at 1 and 3 m. *Compared with normal control, $P < .05$; #Compared with OVX group, $P < .05$; †Compared with OVX + MetS group, $P < .05$. $N = 6$ at each time point in each group. Scale bar indicates 200 μm. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m, 3 months.

induces mitochondrial disruption and swelling in bladder smooth muscle cells.[21] Our study further demonstrates a significant increase in apoptotic cells within the bladder of OVX + MetS rats, highlighting the consequence of ovarian hormone deficiency and HFHS diet induction to lead to apoptosis in bladder cells. This tissue damage is accompanied by upregulation of apoptosis-associated mitochondrial proteins.[25] Our results indicate that OVX + MetS may induce elevated immunoreactivities and mRNA expressions of pro-apoptotic proteins such as Bax, caspase-3, caspase-9, and cytochrome c in the bladder tissue, indicating the activation of mitochondria-dependent apoptotic pathways. Conversely, the expression of anti-apoptotic protein Bcl-2 was significantly reduced. In MetS rats with detrusor overactivity induced by fructose feeding, downregulation of Bcl-2 expression and dysregulation of smoothelin have been observed in detrusor muscle, contributing to increased bladder apoptosis.[17] Furthermore, our study demonstrates that hAFSCs treatment may mitigate apoptosis and improve the expressions of apoptosis-associated mitochondrial proteins, Bcl-2, caspase-3 and caspase-9, in the bladder of OVX + MetS rats. This implies that hAFSCs treatment could potentially inhibit pro-apoptotic signaling pathways.

Stem cell transplantation offers a promising approach for treating MetS-related conditions such as obesity and type 2 diabetes.[34] Preclinical study has demonstrated that administering brown adipose stem cells can effectively reduce body weight and hyperlipidemia.[35] Additionally, umbilical cord stem cells have been shown to inhibit the progression of atherosclerosis and improve conditions such as hyperglycemia, hyperlipidemia, and insulin resistance.[36,37] The current study employed hAFSCs due to their noninvasive origin, being easily obtained from routine amniocentesis. hAFSCs are also advantageous because they can be readily cultured

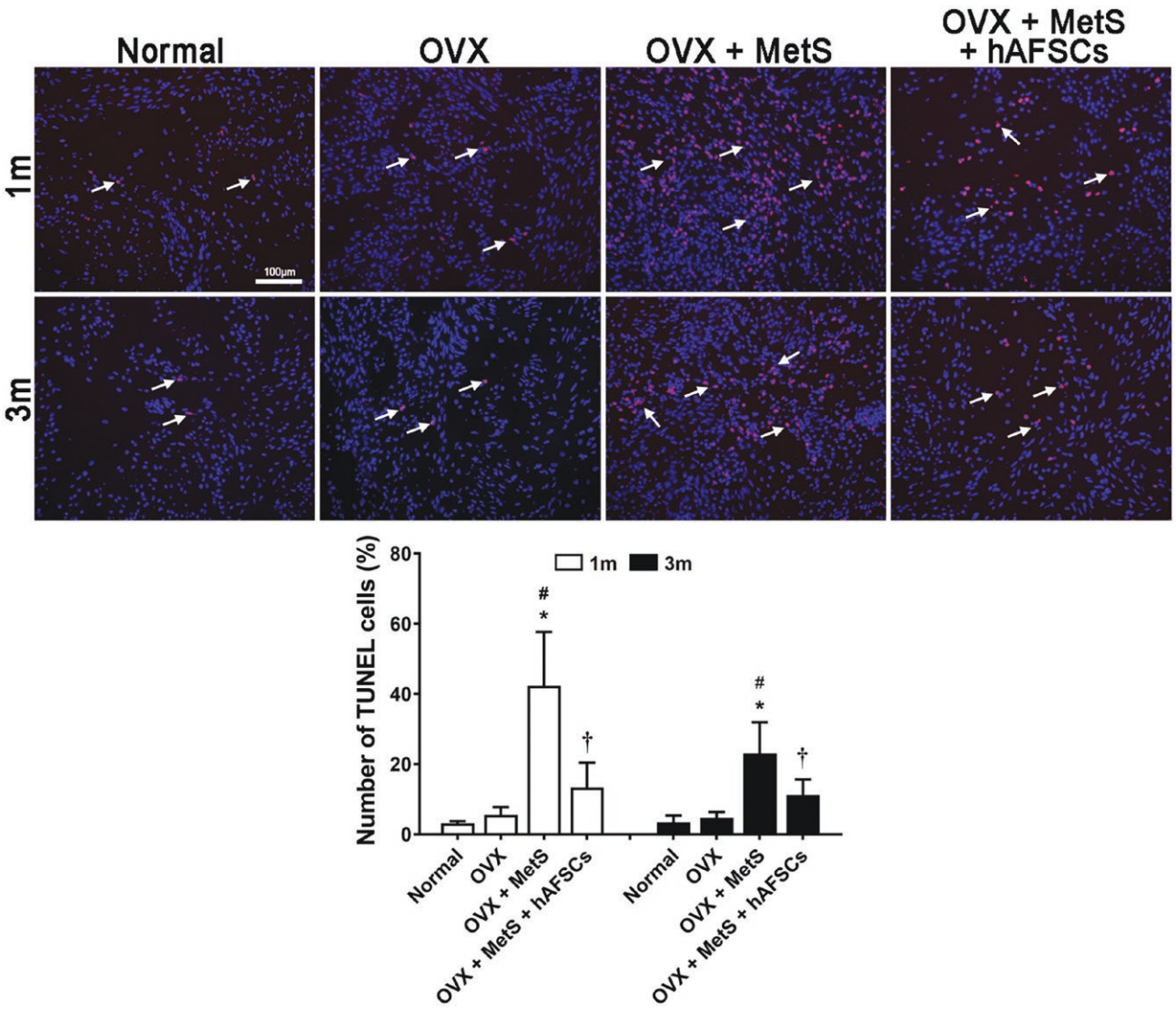


Figure 6. Apoptosis study in the bladder using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Compared with normal control, ovariectomy (OVX) induces nonsignificant increase of apoptotic cells (arrows). Ovariectomized rats with MetS (OVX + MetS) have markedly increased number of apoptotic cells at 1 and 3 m. After hAFSCs treatment, the number of apoptotic cells is significant improved compared with OVX + MetS and recovers to normal control level at 1 and 3 m. *Compared with normal control, $P < .05$; #Compared with OVX group, $P < .05$; †Compared with OVX + MetS group, $P < .05$. $N = 6$ at each time point in each group. Scale bar indicates 100 μm. Statistics: One-way ANOVA with Tukey test. 1 m, 1 month; 3 m, 3 months.

and exhibit phenotypic and genetic stability. Although, this study did not assess immunosuppression, prior animal model studies have indicated that hAFSCs transplantation does not elicit immune rejection.[23,27,28]

This study has limitations since we did not perform OVX + hAFSCs group. First, the main purpose of this study is to compare the differences in cystometric results and changes in molecular parameters related to bladder overactivity between OVX + MetS rats with and without hAFSCs treatment. Second, OVX rats were selected as the operated group to compare the differences between OVX rats with and without Mets. Third, the normal controls were used to compare the differences with OVX (hypoestrogenism) group. Fourth, OVX + hAFSCs group is supposed to compare the effect of MetS on the differences in cystometric results and changes in molecular parameters with OVX + MetS + hAFSCs. However, this comparison could result in a similar comparison between OVX and OVX + MetS. Owing to the above reasons, we did not perform OVX + hAFSCs group. Also, we did not demonstrate the presence of hAFSCs in bladder. According to our previous studies,[27,38,39] rare hCD90-positive hAFSCs could be found at 28 days after hAFSCs treatment. Since our present study examined the difference of cystometric results and the related molecular parameters at 1 and 3 months after hAFSCs treatment, we found that it was very difficult to examine the presence of hAFSCs at these 2 time points. Therefore, we did not intend to demonstrate the presence of injected hAFSCs.

Conclusion

The present study reveals that a combination of HFHS diet and surgical OVX significantly enhances oxidative stress through the mitochondrial pathway over 6 months. Treatment with hAFSCs effectively mitigates bladder inflammation and fibrosis, reduces oxidative stress, and inhibits pro-apoptotic caspase activity, thereby alleviating bladder overactivity in

OVX + MetS rats. Despite the high prevalence of overactive bladder in postmenopausal women with MetS, effective treatments for MetS-induced bladder dysfunction remain insufficient. Our findings suggest the therapeutic potential of hAFSCs for treating bladder dysfunction associated with postmenopausal MetS.

Author contributions

Ching-Chung Liang (Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing), Steven W. Shaw (Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization), Wu-Chiao Hsieh (Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization), Yung-Hsin Huang (Investigation, Resources, Software, Supervision, Validation, Visualization), Chu-Ya Liang (Formal Analysis, Methodology, Resources, Software, Validation, Visualization), and Tsong-Hai Lee (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing).

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Conflict of Interest

All authors declared no potential conflicts of interest.

Data Availability

All relevant data supporting the findings of this study are available within the paper and its supplementary information and from the corresponding author upon reasonable request.

Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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