Basic Study

Human amniotic fluid stem cell therapy can help to regain bladder function similar to insulin treatment in type 2 diabetic rats

Liang CC et al. Stem cells regain diabetic cystopathy
Abstract

BACKGROUND
Diabetes mellitus (DM) is a serious and growing global health burden. It is estimated that 80% of diabetic patients have micturition problems such as poor emptying, urinary incontinence, urgency and urgency incontinence. Patients with diabetic bladder dysfunction are often resistant to currently available therapies. It is necessary to develop new and effective treatment methods.

AIM
To examine human amniotic fluid stem cells (hAFSCs) therapy on bladder dysfunction in a type 2 diabetic rat model.

METHODS
Sixty female Sprague-Dawley rats were divided into 5 groups: Group 1, normal-diet control (control); group 2, high-fat diet (HFD); group 3, HFD plus streptozotocin-induced hyperglycemia like-DM (DM); group 4, DM plus insulin treatment (DM + insulin); group 5, DM plus hAFSCs injection via tail vein (DM + hAFSCs). Conscious cystometric studies were done at 4 and 12 wk after insulin or hAFSCs treatment including peak voiding pressure, voided volume, intercontraction interval, bladder capacity and residual volume. Immunoreactivities and/or mRNA expressions of muscarinic receptors, nerve growth factor (NGF), sensory nerve markers in bladder, and insulin, MafA, and pancreatic-duodenal homeobox-1 (PDX-1) in pancreatic beta cells were studied.

RESULTS
Compared with DM rats, insulin but not hAFSCs treatment could reduce the bladder weight and improve the voided volume, intercontraction interval, bladder capacity and residual volume (P < 0.05). However, both insulin and hAFSCs treatment could help to regain the blood glucose and bladder functions to the levels near controls (P > 0.05). The
immunoreactivities and mRNA of M2- and M3-muscarinic receptor (M2 and M3) were increased mainly at 4 wk ($P < 0.05$), while the number of beta cells in islets and the immunoreactivities and/or mRNA of NGF, calcitonin gene-related peptide (CGRP), substance P, insulin, MafA and PDX-1 were decreased in DM rats ($P < 0.05$). However, insulin and hAFSCs treatment could help to regain the expressions of M2, M3, NGF, CGRP, substance P, MafA and PDX-1 to near the levels of controls at 4 and/or 12 wk ($P > 0.05$).

CONCLUSION
Insulin but not hAFSCs therapy can recover the bladder dysfunction caused by DM, however, hAFSCs and insulin therapy can help to regain bladder function to near the levels of control.

Key Words: Amniotic fluid; Bladder dysfunction; Diabetes; Insulin; Stem cells

Liang CC, Shaw SW, Huang YH, Lee TH. Human amniotic fluid stem cell therapy can help to regain bladder function similar to insulin treatment in type 2 diabetic rats . World J Stem Cells 2022; In press

Core Tip: Diabetic patients with bladder dysfunction are often resistant to currently available therapies. Stem cells demonstrate the efficacy in preclinical studies of diabetic bladder dysfunction. Human amniotic fluid stem cells (hAFSCs) can be obtained from amniotic fluid, and phenotypically and genetically stable, indicating that hAFSCs can be used as a novel source of cell therapy. Here, we demonstrated although it is insulin but not hAFSCs therapy that can help recovering the bladder dysfunction caused by diabetes mellitus (DM), both insulin and hAFSCs treatment can help to regain bladder function to near the levels of control. Our study highlights the potential of hAFSCs for cell replacement and regeneration therapy for DM.
INTRODUCTION

Diabetes mellitus (DM) is a serious and growing global health burden. During the past few decades, the prevalence of DM has increased significantly, mainly due to the continuous rise in the incidence of type 2 DM[1]. Type 2 DM is characterized by defects in insulin secreted by pancreatic beta cells and peripheral insulin resistance[2]. As the disease progresses, insulin deficiency may appear. Exogenous insulin has been used to prevent various complications of DM, but almost all patients will eventually have complications, including obesity, nephropathy, neuropathy and cardiovascular disease[3]. This may be due to the failure of exogenous insulin to regulate glucose levels as effectively as that provided by endogenous insulin from beta cells[4]. Beta cells in the pancreatic islets are the only cell type that secretes insulin in response to blood glucose, but how to maintain the physiological function of beta cells through insulin transcription factors is not yet fully understood.

Among individuals diagnosed with DM, 80% have micturition problems such as poor emptying, urinary incontinence, urgency and urgency incontinence[5]. Studies have shown that the pathophysiology of diabetic bladder dysfunction is multifactorial[6]. In a streptozotocin (STZ)-induced diabetic rat model, STZ-induced hyperglycemia like-DM may act through an oxidative stress mechanism that affects multiple tissues including innervation, ganglia, and urinary tree, and there are reduced nerve growth factor (NGF) levels in the bladder and dorsal root ganglia of lumbar spine which are associated with voiding dysfunction caused by defects in A-delta and C fiber bladder afferent nerves[6,7]. The antioxidant defense at the median- and long-time can participate in recovering tissue functions, even structural changes. Also, up-regulation of bladder muscarinic receptors can lead to bladder overactivity in STZ-induced hyperglycemia like-diabetic rats[8,9].

Patients with diabetic bladder dysfunction are often resistant to currently available therapies. Therefore, it is necessary to develop new and effective treatment methods. Stem cells have recently demonstrated efficacy in preclinical studies of diabetic bladder dysfunction[10,11]. Human amniotic fluid stem cells (hAFSCs) can be obtained from
amniotic fluid, easy to culture, and phenotypically and genetically stable, indicating that these stem cells can be used as a novel source of cell therapy\[^{12}\]. The present study aims to investigate the effect of hAFSCs therapy and whether the therapeutic effect could be similar to insulin treatment using a type 2 DM rat model.

**MATERIALS AND METHODS**

*Animal model*

Female Sprague-Dawley rats (10-12 wk old) were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 wk prior to experiment. Sixty rats were divided into 5 groups: Group 1, normal-diet control (control) with rodent chow diet (LabDiet 5001, Richmond, IN, United States) containing 13.6% fat, 28.9% protein and 57.5% carbohydrate (Kcal); group 2, high-fat diet (HFD) (D12492, Research Diets, New Brunswick, NJ, United States\[^{13}\]) containing 60% fat, 20% protein and 20% carbohydrate (Kcal); group 3, HFD plus STZ-induced hyperglycemia like-DM (DM), simulating human type 2 DM\[^{14}\]; group 4, hyperglycemia like-DM plus insulin treatment (DM + insulin); group 5, hyperglycemia like-DM plus hAFSCs injection via tail vein (DM + hAFSCs).

All rats received bladder function test using conscious cystometry at 4 and 12 wk after insulin treatment or hAFSCs therapy (\(n = 6\) in each time point). Expressions of NGF and M2- and M3-muscarinic receptor (M2 and M3) were measured by immunohistochemistry and real-time polymerase chain reaction (RT-PCR). The expressions of calcitonin gene-related peptide (CGRP) and substance P in bladder and the expressions of insulin, Maf family of transcription factors (MafA) and pancreatic-duodenal homeobox-1 (PDX-1) in pancreatic beta cells were measured by immunohistochemistry. The experimental procedure is shown in Figure 1. All appropriate measures were taken to minimize pain or discomfort. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.
The reasons why the present study used female rats are first, our previous study\textsuperscript{[11]} examined the protective effect of hAFSCs against bladder dysfunction in a rat model of type 1 DM. We found type 1 DM could cause bladder dysfunction, such as more frequent non-voiding bladder contraction, longer intercontraction interval and larger voided and residual volume compared with controls after the induction of DM in female rats. In order to investigate if the results of bladder dysfunction in type 2 DM rats could be similar to those in type 1 DM rats, we used the same female rats for comparison. Second, authors are clinicians and experts in female urology and obstetrics, the use of female rats in animal studies was to understand and solve the similar urological problems in female DM patients.

\textit{Induction of hyperglycemia like-DM}

The DM rats were first fed with a HFD (60\% kcal fat, D12492, Research Diets, New Brunswick, NJ, United States) and water ad libitum. After 4 wk of dietary manipulation, rats were then pretreated with a single intraperitoneal dose of 35 mg/kg STZ, dissolved in 0.1 M citrate buffer with pH 4.5 to induce experimental hyperglycemia like-DM\textsuperscript{[8,15]}, which resembles the condition of human type 2 DM. Fasting blood glucose levels were determined 72 h later after STZ injection by a glucometer (ACCU-CHEK Active, Roche Diagnostics, Germany) from tail blood. The rats with fasting blood glucose 300 mg/dL or greater were considered as diabetic\textsuperscript{[8,16]}. The HFD group was injected only with citrate buffer intraperitoneally. The control was given a regular diet and did not receive any treatment. The rats were allowed to continue feeding on their respective diets until euthanasia.

The DM rats were administered with long-acting glargine insulin (LANTUS\textsuperscript{®}, Sanofi-Aventis, Germany) at a dose of 3 U/d subcutaneously from 7 d after STZ injection, and the dose was later adjusted according to the glycemic level\textsuperscript{[17]}. The DM + insulin rats received insulin injection at a fixed time (9:00 AM) every day until euthanasia.

\textit{Isolation and characterization of hAFSCs for therapy}
The hAFSCs were obtained by routine amniocentesis from six healthy pregnant donors in 15-20 gestational weeks. Cells were cultured in the StemPro® mesenchymal stem cell (MSC) serum free medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, United States) and incubated at 37 °C with 5% carbon dioxide. The specific surface antigens of hAFSCs were characterized by flow cytometry analyses as shown in previous work[18]. The cultured cells were trypsinized and stained with phycoerythrin-conjugated antibodies against CD44, CD73, CD90, CD105, CD117 and CD45 (BD PharMingen, CA, United States). Thereafter, the cells were analyzed using the Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Passage 4-6 hAFSCs were collected and prepared to a final concentration of $3 \times 10^6$ cells/mL phosphate buffered saline. In DM + hAFSCs group, single dose of $3 \times 10^6$ hAFSCs were injected intravenously via tail vein at 7 d after hyperglycemia like-DM induction. The treatment dose of hAFSCs was determined according to the previous study that used adipose tissue-derived stem cells to treat bladder dysfunction of STZ-diabetic rats[10].

**Cystometric study**

All rats received suprapubic tube implantation under 1.5% isoflurane inhalation 2 d prior to implementation of cystometry. The animals were placed in special metabolic cages (Med Associates, Saint Albans, VT, United States) to perform conscious cystometric studies at 4 and 12 wk after insulin treatment or hAFSCs therapy according to our previous study[19]. Five cystometric variables were investigated including peak voiding pressure, voided volume, intercontraction interval, bladder capacity and residual volume[20]. Cystometry Analysis Version 1.05 (Catamount Research and Development) was used for cystometric analysis.

**Immunohistochemistry**

The rats were euthanized immediately after cystometry, and their urinary bladders and pancreases were harvested for the histological and microbiological examinations at 4 and 12 wk after insulin treatment or hAFSCs therapy. The dissected bladders and
pancreases were fixed in optimal cutting temperature compound, frozen in powdered dry ice and stored at -80 °C. Then, they were subjected to cryosections with 12-μm thickness at -20 °C, and the sections were transferred to glass microscope slides coated with saline (Muto Pure Chemical, Tokyo, Japan).

The immunostaining against NGF, M2, M3, CGRP and substance P in bladder sections and against insulin, MafA and PDX-1 in pancreas sections was performed with avidin-biotin peroxidase method. First, the sections were fixed for 10 min in acetone for NGF, CGRP, substance P and PDX-1, and 10 min in 4% paraformaldehyde for M2, M3, insulin and MafA, air dried and then rinsed with phosphate buffered saline. After blocking with Dako REAL peroxidase blocking solution (code S2023, DAKO Corp, Carpinteria, CA, United States) for 20 min, the sections were washed and incubated for 18-20 h at 4 °C with a rabbit polyclonal antibody against NGF (1:700, OriGene Technologies, Inc., Rockville, MD, United States), M2 (1:700, Millipore, Temecula, CA, United States), M3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States), MafA (1:200, Abcam, Cambridge, MA, United States) and PDX-1 (1:100, Abcam, Cambridge, MA, United States), a goat polyclonal antibody against CGRP (1:200, Abcam, Cambridge, MA, United States), and a mouse monoclonal antibody against substance P (1:75, Abcam, Cambridge, MA, United States) and insulin (1:1200, Thermo Fisher Scientific Inc., Waltham, MA, United States). Then, the sections were washed and incubated for one hour using biotinylated secondary antibodies at 1:500 dilution (Vector Laboratories, Burlingame, CA, United States). Staining was developed with 3,3'-diaminobenzidine plus hydrogen peroxide as the chromogen, and the pancreas sections were counterstained with hematoxylin. Negative controls were prepared from the same tissue blocks by omitting the specific primary antibodies and using normal, non-immune serum supernatant from the same sources.

Tissue slides were examined under Olympus BX-51 microscope. Immunoreactivities were analyzed using Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, United States). The immunoreactivity ratio using the optical density of diabetic rats with or without insulin and hAFSCs treatment to that of control rats was determined.
The following pancreas parameters were assessed\cite{21}: (1) The area percentage with a positive anti-insulin antibody reaction in pancreas islet was measured in 5 different microscopic fields per islet and 5 islets per rat with a total of 150 microscopic fields measured in each time point \((n = 6\) in each time point); (2) The percentage of beta cells per islet was calculated by counting the number of beta cell nuclei \((B)\) and the total number of islet cell nuclei per islet profile \((I)\) to determine the percentage of beta cell per total islet cell \((Beta-p)\) with the equation: \(Beta-p = \frac{(B/I)}{100}\). The Beta-p was calculated in 4 islets per pancreas in each rat with a total of 24 islets in each time point; and (3) Average area was determined in 4 islets per pancreas in each rat with a total of 24 islets in each time point.

**RT-PCR**

RT-PCR was carried out according to the manufacturer’s protocol. The total RNAs were prepared using a Trizol reagent (Invitrogen, Carlsbad, CA, United States) and incubated in reverse transcription mixture at 25 °C for 5 min, 50 °C for one hour, 70 °C for 15 min, and finally, the tubes were cooled to 4 °C for 5 min. The gene expression for NGF, M2, M3, CGRP and substance P in the bladder tissue was analyzed by RT-PCR using the inventoried TaqMan assays from Applied Biosystems (Life Technologies, Grand Island, NY, United States). The assay codes of NGF, M2, M3, CGRP and substance P were Rn01533872-m1, Rn02532311-s1, Rn00560986-s1, Rn01511353-g1 and Rn01500392-m1, respectively (Applied Biosystems, Oster City, CA, United States) (Table 1). The GAPDH assay code (Rs99999916-s1, Table 1) was used as an endogenous control to allow for semi-quantification of relative gene expression. Thermal cycling and fluorescence detection were performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Oster City, CA, United States). PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for one minute. The data were calculated using the \(2^{[-\Delta C(T)]}\) method\cite{22}. A ratio of the mRNA level of DM rats with or without hAFSCs therapy to that of control rats was determined. The values were summated and expressed as mean ± SD and were compared statistically among...
the three groups of control, DM, and DM + hAFSCs groups and among different time points in each group.

**Statistical analysis**

Data were analyzed with Prism 5 (GraphPad Software Inc., San Diego, CA, United States) and expressed as median with first and third quartile for continuous variables. First, two-way analysis of variance was used for analysis. Then, Kruskal-Wallis test with posthoc Bonferroni test was performed for intergroup analysis. Mann-Whitney U test was used for the comparison between 4 wk and 12 wk. Probability values of $< 0.05$ were considered statistically significant. The statistical review of the study was performed by a biomedical statistician.

**RESULTS**

**hAFSCs therapy does not improve blood glucose and bladder weight**

The DM and DM + hAFSCs rats had significantly increased levels of blood glucose at 4 and 12 wk and significantly decreased body weight at 12 wk compared with normal control and HFD control ($P < 0.05$), and significantly increased bladder weight compared with HFD control at 4 and/or 12 wk ($P < 0.05$). The DM + insulin rats had similar levels of blood glucose and similar body and bladder weight compared with normal control and HFD control at 4 and 12 wk ($P > 0.05$), but there was significantly reduced bladder weight compared with DM rats at 4 and 12 wk ($P < 0.05$). However, DM + hAFSCs rats did not show significantly reduced body weight, blood glucose levels and bladder weight compared with DM rats ($P > 0.05$) but there was significantly increased bladder weight compared with DM + insulin rats ($P < 0.05$) (Table 2).

**Bladder dysfunctions in DM rats are improved after insulin treatment and hAFSCs therapy**

Similar to our previous study in type 1 DM rats[11], the type 2 DM rats had significant increase in peak voided volume, intercontraction interval, bladder capacity and residual
volume at 4 and/or 12 wk after DM induction when compared with ND and HFD controls ($P < 0.05$). These bladder dysfunctions were improved after insulin treatment ($P < 0.05$) but not after hAFSCs therapy ($P > 0.05$). The DM + hAFSCs rats had significantly higher peak voided volume, longer intercontraction interval, larger bladder capacity and more residual volume than DM + insulin rats mainly at 4 wk with higher peak voided volume also at 12 wk ($P < 0.05$) (Figure 2). However, both insulin and hAFSCs treatment could help to regain the bladder functions to the levels near controls ($P > 0.05$).

**Expressions of M2, M3, NGF, CGRP and substance P are improved after insulin treatment and hAFSCs therapy**

The DM rats had significantly increased expressions of M3 mRNA and M2 immunoreactivity at 4 wk compared with 12 wk ($P < 0.05$). Also, there were increased M2 and M3 mRNAs and M3 immunoreactivity at 4 wk and M2 immunoreactivity at 4 and 12 wk compared with ND/HFD controls ($P < 0.05$). However, M3 mRNA and M2 immunoreactivity recovered in DM + insulin rats at 4 wk after DM induction ($P < 0.05$). In contrast, DM rats had significantly decreased mRNA of NGF and substance P (a sensory nerve marker of C fiber) at 4 and/or 12 wk compared with ND/HFD controls ($P < 0.05$).

The DM rats had also significantly reduced immunoreactivities of NGF, CGRP (a sensory nerve marker of A-delta fiber) and substance P at 4 and 12 wk compared with ND/HFD controls ($P < 0.05$). Insulin treatment could improve mRNA of NGF and substance P and immunoreactivity of CGRP at 4 wk, and hAFSCs treatment could improve mRNA and immunoreactivity of NGF at 4 wk after DM induction ($P < 0.05$). However, both insulin and hAFSCs treatment could help to regain the expressions of M2, M3, NGF, CGRP, and/or substance P to near the levels of controls at 4 and/or 12 wk ($P > 0.05$) (Figures 3 and 4, Supplemental Figures 1-5).
**Insulin treatment and hAFSCs therapy can increase insulin, MafA and PDX-1 expression**

STZ-induced degenerative changes in beta cells lead to a decrease in the immunoreactivities of insulin, MafA and PDX-1 in the pancreatic islets at 4 and/or 12 wk ($P < 0.05$). There were also decreased beta cells per total number of islets cells, decreased area of reactive beta cell and decreased average area of islets at 4 and 12 wk in DM rats ($P < 0.05$). However, insulin treatment could recover MafA immunoreactivity at 4 wk, the average area of islets at 12 wk and the area of reactive beta cell and PDX-1 immunoreactivity at 4 and 12 wk ($P < 0.05$). To a less extent, hAFSCs treatment could only recover PDX-1 immunoreactivity at 4/12 wk after DM induction ($P < 0.05$). However, both insulin and hAFSCs treatment could help to regain the expressions of MafA and PDX-1 to near the levels of controls at 4 and/or 12 wk ($P > 0.05$) (Figures 5 and 6).

**DISCUSSION**

Contrast to previous literatures that explored bladder hypertrophy mostly using type 1 DM model\cite{23}, our study investigated bladder dysfunction using type 2 DM model. A systemic review demonstrated that insulin treatment starting early after STZ injection could prevent bladder hypertrophy, assessed by bladder weight\cite{24}. Our results indicated that STZ-induced hyperglycemia like-DM rats had increased bladder weight and blood glucose levels, but bladder weight could be recovered and blood sugar could be regained to near the levels of controls by daily insulin treatment for 4 and 12 wk starting one week after DM induction.

In addition to lowering blood glucose level, the immediate insulin treatment after STZ injection is reported to improve bladder dysfunction, including micturition frequency, voided volume and bladder capacity\cite{25,26}. In Zucker diabetic fatty rats, a hereditary type 2 DM model, the cystometric studies exhibited a high voided volume with an increased bladder capacity and residual volume\cite{27,28}. Christ et al\cite{29} used a rat model with low-dose STZ treatment plus no HFD and found insulin treatment could
improve several cystometric variables including micturition volume, bladder capacity, residual volume and micturition pressure in diabetic bladder dysfunction. In the present study, we adopted a rat model that combined HFD and low-dose STZ treatment simulating the human type 2 DM\textsuperscript{[14]}. Our cystometric study demonstrated that DM rats had a significant increase in the peak voided volume, intercontraction interval, bladder capacity and residual volume at 4 and/or 12 wk after DM induction, and these bladder dysfunctions could be recovered after insulin treatment but not hAFSCs therapy. However, both insulin and hAFSCs treatment could help to regain the bladder functions to the levels near controls.

The present study further demonstrated that although both insulin treatment and hAFSCs therapy could help to regain bladder function (Figures 2-5), the effect of insulin treatment was better than hAFSCs therapy. As shown in Table 2, compared with hAFSCs therapy, insulin treatment could reach a better bladder weight recovery at 4 and 12 wk which could be helpful to improve bladder dysfunction. As to stem cell, previous study had demonstrated that therapy of human umbilical cord blood-derived cells into DM mice did not lead to improvement in hyperglycemia\textsuperscript{[90]}. Also, therapy of adipose tissue-derived stem cells into STZ-induced hyperglycemia like-DM rats could ameliorate diabetic bladder dysfunctions without changing the hyperglycemia in DM mice\textsuperscript{[10]}. Our previous studies has also shown bladder dysfunction can be improved by hAFSCs transplantation into bladder in STZ-induced hyperglycemia like-DM rats\textsuperscript{[11]} and at the nerve injury site in rats with pelvic nerve transection\textsuperscript{[31]}. Although stem cell therapy is reported to be effective in treating type 2 DM through the effect by reducing insulin dose and HbA1c levels\textsuperscript{[32]}, single injection of hAFSCs in the present study could be not effective enough compared with daily regular insulin injection to induce adequate reduction of blood glucose level.

The age-related changes in bladder function have revealed significant inter-individual variability and sex-related disparity. Aged mice were prone to demonstrate voiding and storage dysfunctions resembling to detrusor hyperactivity with impaired contractility, which were more common and severe in males\textsuperscript{[33]}. The mRNA expression
of M3 muscarinic receptor was found being reduced in the bladder tissue of aged males and the β2-adrenoceptors in aged females[33]. Previous study suggested that the transition from compensated to decompensated bladder dysfunction occurs 9-12 wk after DM induction in mice by STZ[34]. However, Xiao et al[4] found that there was no significant difference of voided volume and bladder capacity at 3 and 11 wk in STZ-induced hyperglycemia like-DM rats. The present study showed that the peak voiding pressure, peak voided volume, intercontraction interval, bladder capacity, and residual volume did not show difference between 4 and 12 wk after DM induction. However, the M3 mRNA and M2 immunoreactivity were significantly reduced at 12 wk compared with that at 4 wk in DM rats but not in DM + insulin and DM + hAFSCs rats. It is possible that the present study period could be too short to show the significant difference of bladder dysfunction related to age. Future study may be needed to investigate the influence of age on diabetic bladder dysfunction.

In addition to bladder smooth muscle cell and urothelial dysfunction, diabetic bladder dysfunction can also be associated with the physiological changes in neuronal degeneration[6]. It has been reported that DM decreases the activity of A-delta and C fiber of bladder afferent pathways, which is related to the reduction of NGF production in bladder[7]. In the present study, CGRP immunoreactivity, a sensory nerve marker of A-delta fiber, at 4 and 12 wk and substance P immunoreactivity/mRNA, a sensory nerve marker of C fiber, at 4 and/or 12 wk were decreased, reflecting the presence of peripheral neuropathy in DM rats. These neuropathic changes may lead to the loss of neurotrophic support and detrusor contraction dysfunction. At 4 wk after insulin treatment or hAFSCs therapy, the immunoreactivities and/or mRNA expressions of some NGF, CGRP and substance P improved significantly in the diabetic bladder, indicating the possibility that the production of NGF in bladder and/or the transport of NGF to bladder afferent pathway were restored.

The motor and sensory functions of bladder muscarinic mechanism are thought to be involved in the alternation of bladder function in STZ-induced hyperglycemia like-DM rats[9,35]. Previous studies demonstrated that 8 wk of STZ-induced hyperglycemia like-
DM could result in an increased density of muscarinic receptors in the bladder of rats, and insulin treatment starting early after DM induction could prevent the up-regulation of muscarinic receptors\textsuperscript{56}. The present results showed that the M2/M3 mRNA at 4 wk and M2/M3 immunoreactivities at 4 and/or 12 wk increased significantly in DM rats. However, M3 mRNA and M2 immunoreactivity could be recovered in DM + insulin rats at 4 wk after DM induction, and both insulin and hAFSCs treatment could help to regain the expressions of M2 and M3 to near the levels of controls at 4 and/or 12 wk.

In normal condition, elevated glucose levels can stimulate pancreatic beta cells to secrete insulin. However, the number of beta cells is reduced with insufficient insulin secretion in DM, and the glucose-beta cell reaction will be impaired\textsuperscript{57}. Previous study had shown that using a high-dose STZ-induced hyperglycemia like-DM rat model, STZ-induced degenerative changes could cause insulin resistance development, chronic glucose and lipid metabolism changes, multi-organ deterioration, and, finally, beta-cell exhausting, which would result in a reduced number of beta cells and insulin immunoreactivity in pancreatic islets\textsuperscript{21}. The present study demonstrated that the significant reduction in the size of pancreatic islets and the number of beta cells might lead to a decrease in the immunoreactivity of insulin in the pancreatic islets of DM rats.

The present study showed the immunoreactivities of MafA and PDX-1 in pancreatic islets of type 2 DM rats were decreased. However, both insulin treatment and hAFSCs therapy could recover PDX-1 immunoreactivity at 4 and 12 wk and insulin treatment could recover MafA immunoreactivity at 4 wk after DM induction. Also, both insulin and hAFSCs treatment could help to regain the expressions of MafA and PDX-1 to near the levels of controls at 4 and/or 12 wk. It has been reported that MafA is a beta cell-specific transcription factor and a key regulator of glucose stimulated insulin secretion\textsuperscript{38,39}. PDX-1 plays a crucial role in the differentiation of pancreatic beta cells and the regulation of insulin and several beta cell-related genes to maintain cellular function\textsuperscript{38}. The expression of PDX-1 is suggested being regulated by MafA in beta cells\textsuperscript{40}. Under the diabetic conditions, high glucose levels may reduce the DNA binding activities of PDX-1 and MafA by triggering oxidative stress, which results in the
suppression of insulin biosynthesis and secretion. It is possible that MafA and PDX-1 may also act as important regulators of glucose-stimulated insulin secretion in the pancreas of our type 2 DM model.

It is well known that long-term medication to reduce blood glucose is costly and lacks satisfactory effect, and there is a low life expectancy in DM patients. Although there are many antidiabetic agents for type 2 DM, medications including insulin usually result in some side-effects such as weight gain and gastrointestinal distress. Stem cell treatment is found to significantly reduce insulin dose in DM patients studied, so it can act as a substitute for DM patients who had islet cell dysfunction with failure in ideal blood glucose control, despite the use of high dose of insulin. Previous study has shown that amniotic fluid-derived MSCs could secret NGF and brain-derived neurotrophic factor. The present study showed that hAFSCs therapy could improve the expressions of NGF immunoreactivity and mRNA mainly at 4 wk after DM induction. It is possible the NGF secreted by hAFSCs may help to enhance the neurotrophic effect to improve tissue cell regeneration by inhibiting bladder cell apoptosis in diabetic rats and suggests that hAFSCs could be a potential cell source for cell replacement and regeneration therapy for DM.

However, there are some limitations. First, we only used single dose at single time point to investigate the effect of hAFSCs on diabetic bladder dysfunction. It is possible that different doses and different time points of stem cell therapy may have better effects on diabetic bladder function. Second, we examined the functional and morphological alterations of diabetic bladder at 4 and 12 wk after DM induction. It is possible to obtain better results if the effects at longer time points were examined. Third, we did not directly examine the cellular differentiation of hAFSCs therapy in bladder and pancreas. Further studies are needed to investigate the mechanisms of hAFSCs therapy to improve bladder dysfunction in type 2 DM.

CONCLUSION
The present results show that although insulin treatment is more effective than hAFSCs therapy, both may help to regain the bladder function to near the levels of control after STZ-induced hyperglycemia like-diabetes.

**ARTICLE HIGHLIGHTS**

*Research background*
Diabetes mellitus (DM) is a serious and growing global health burden. It is estimated that 80% of diabetic patients have micturition problems such as poor emptying, urinary incontinence, urgency and urgency incontinence. Patients with diabetic bladder dysfunction are often resistant to currently available therapies. It is necessary to develop new and effective treatment methods.

*Research motivation*
Exogenous insulin has been used to prevent various complications of DM, but almost all patients will eventually have complications. Stem cells have recently demonstrated efficacy in preclinical studies of diabetic bladder dysfunction. Human amniotic fluid stem cells (hAFSCs) can be obtained from amniotic fluid, easy to culture, and phenotypically and genetically stable, suggesting that these stem cells can be used as a novel source of cell therapy.

*Research objectives*
The present study aims to investigate the effect of hAFSCs therapy and whether the therapeutic effect could be similar to insulin treatment on bladder dysfunction using a type 2 DM rat model.

*Research methods*
Sixty female Sprague-Dawley rats were divided into 5 groups: Group 1, normal-diet control (control); group 2, high-fat diet (HFD); group 3, HFD plus streptozotocin-induced hyperglycemia like-DM (DM); group 4, DM plus insulin treatment (DM +
insulin); group 5, DM plus hAFSCs injection via tail vein (DM + hAFSCs). Conscious cystometric studies were done at 4 and 12 wk after insulin treatment or 3 × 10^6 hAFSCs therapy. Immunoreactivities and mRNA expressions of bladder muscarinic receptors, nerve growth factor (NGF), sensory nerve markers, insulin, MafA, and pancreatic-duodenal homeobox-1 (PDX-1) in pancreatic beta cells were studied.

Research results
Compared with DM rats, insulin but not hAFSCs treatment could reduce the bladder weight and improve the voided volume, intercontraction interval, bladder capacity and residual volume. However, both insulin and hAFSCs treatment could help to regain the blood glucose and bladder functions to the levels near controls. The immunoreactivities and mRNA of M2- and M3-muscarinic receptor (M2 and M3) were increased mainly at 4 wk, while the number of beta cells in islets and the immunoreactivities and/or mRNA of NGF, calcitonin gene-related peptide (CGRP), substance P, insulin, MafA and PDX-1 were decreased in DM rats. However, both insulin and hAFSCs treatment could help to regain the expressions of M2, M3, NGF, CGRP, substance P, MafA and PDX-1 to near the levels of controls at 4 and/or 12 wk.

Research conclusions
Although insulin but not hAFSCs therapy can recover the bladder dysfunction caused by type 2 DM, both insulin and hAFSCs therapy can help to regain the bladder function to near the levels of control.

Research perspectives
In streptozotocin-induced diabetic rat model, hAFSCs therapy could help to regain bladder function and may serve as an alternative treatment for diabetic bladder dysfunction. Our study highlights the potential of hAFSCs for cell replacement and regeneration therapy for DM.
<table>
<thead>
<tr>
<th>Primary Source</th>
<th>Title and Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a></td>
</tr>
<tr>
<td>2</td>
<td>citeseerx.ist.psu.edu</td>
</tr>
<tr>
<td>3</td>
<td>pt.scribd.com</td>
</tr>
<tr>
<td>4</td>
<td>Wei-Hsuan Hsu, Bao-Hong Lee, Chih-Heng Li, Ya-Wen Hsu, Tzu-Ming Pan. &quot;Monascin and AITC Attenuate Methylglyoxal-Induced PPARy Phosphorylation and Degradation through Inhibition of the Oxidative Stress/PKC Pathway Depending on Nrf2 Activation&quot;, Journal of Agricultural and Food Chemistry, 2013</td>
</tr>
<tr>
<td>5</td>
<td>f6publishing.blob.core.windows.net</td>
</tr>
<tr>
<td>7</td>
<td>Siham K. Abunasef, Hanan A. Amin, Ghada A. Abdel-Hamid. &quot;A histological and...</td>
</tr>
</tbody>
</table>
immunohistochemical study of beta cells in streptozotocin diabetic rats treated with caffeine", Folia Histochemica et Cytobiologica, 2014
Crossref