Neonatal Bladder-derived Mesenchymal Stem Cells Ameliorate Bladder Dysfunction in Diabetic Rat Models

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Purpose: To evaluate the effect of a new mesenchymal stem cell type derived from the neonatal bladder (nMSC-B) on diabetic bladder dysfunction (DBD).

Materials and Methods: nMSC-B were harvested from neonatal male Sprague-Dawley rat's bladder and expanded in culture. nMSC-B were transferred to Type-1 diabetic rats which were induced by a single dose 45 mg/kg Streptozocin (STZ). Stem cells were transferred via intraperitoneally (IP) (DM-IP group, n:6) and by direct injection to the detrusor (DM-D group, n:6) at 12th week following diabetes and compared with Phosphate Buffered Saline (PBS) injected diabetic rats (DM-PBS group, n:6) and age-matched PBS injected non-diabetic normal rats (NR-PBS group, n:6). All rats were evaluated histopathologically and functionally four weeks after the stem cell treatment.

Results: nMSC-B showed improvement in both voiding function and bladder structure. The maximum voiding pressure (MVP) values in the DM-PBS group were lower compare to DM-IP, DM-D and NR-PBS groups (13.27 ± 0.78 vs 16.27 ± 0.61, 28.59 ± 2.09, 21.54 ± 1.00, respectively, $P < .001$). There was a significant improvement for MVP values in stem cell-treated groups. Immunohistochemical examination revealed decreased bladder smooth muscle (SM), increased fibrosis and desquamation in urothelia in diabetic groups compared to normal group($P < .001$). We detected recovery in the stem cell groups. This recovery was more evident in DM-D group. No statistical difference was observed in SM and fibrosis between DM-D and NR-PBS groups ($P = .9$).

Conclusion: It was shown that nMSCBs provided amelioration of DBD. We think that nMSC-B constitutes an effective treatment method in DBD.

Keywords: conscious cystometry; diabetic bladder dysfunction; mesenchymal stem cell; underactive bladder

INTRODUCTION

Diabetes mellitus (DM) is a common chronic disease occurring due to partial or complete lack of insulin, leading to multiple complications and related morbidities, and causing high health expenditure with the number of diabetic patients increasing every day. Urinary system complications are the most expensive complications of diabetes.[1] Diabetic Bladder Dysfunction (DBD) occurs in 43% to 87% of insulin-dependent diabetics, with no sex or age differences. Although it is the most common complication, it is the least known DM complication.[2,3]

Although blood sugar levels can be kept under control and symptomatic treatments are available, none of these are satisfactory for DBD treatment. Recently, cellular-based treatments based on animal models are considered among promising treatment options.[4,5] Mesenchymal Stem Cells (MSCs) are among the most frequently used stem cell types due to their easy obtainability from many tissues and organs, high differentiation potential, rapid reproduction and fusion abilities, contribution to function and development of all tissue cells, as a supporting cell, ability to repair cell and/or tissues through the release of cytokines and growth factors, and the lack of a need for tissue compatibility since they have an immunosuppressive effect and low

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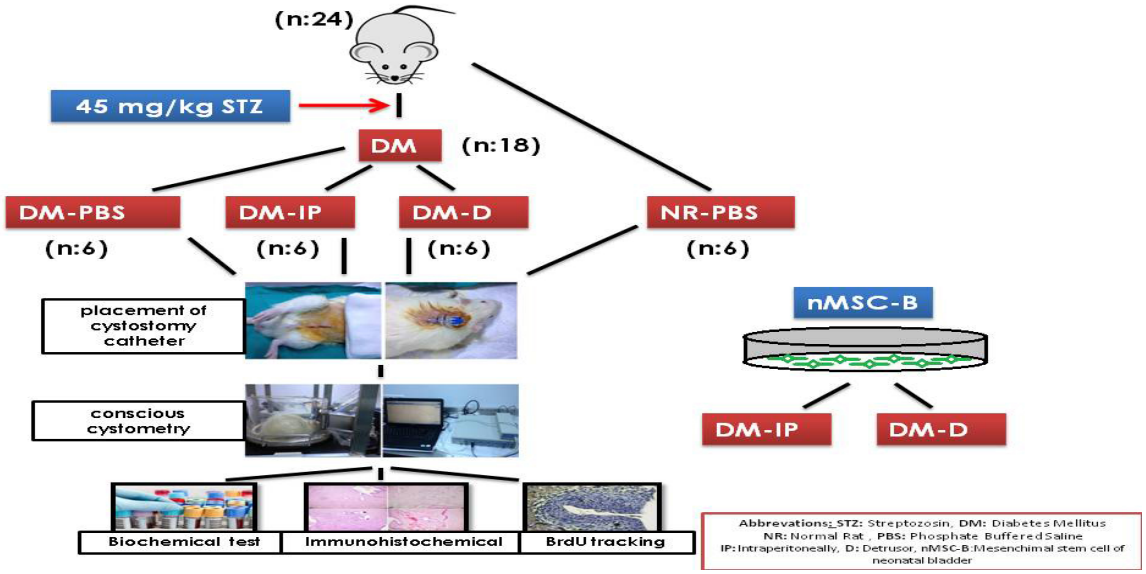

Figure 1. The schematic representation of the experimental procedure

immunogenicity.[6-8]
In the current study, we aimed to investigate the efficacy of Neonatal bladder-derived mesenchymal stem cells (nMSC-B) which have not yet been used before, in the treatment of DBD.

MATERIALS AND METHODS

Study Design and Formation of the Groups

Ethical Board Consent was taken from Animal Experimental Studies Ethical Board with the number 2013/64 for this study.
A total of 24 twelve-week old male Sprague-Dawley type rats (480-540 gr) were used for the study. Rats were kept in rooms organized to provide 12 hours of

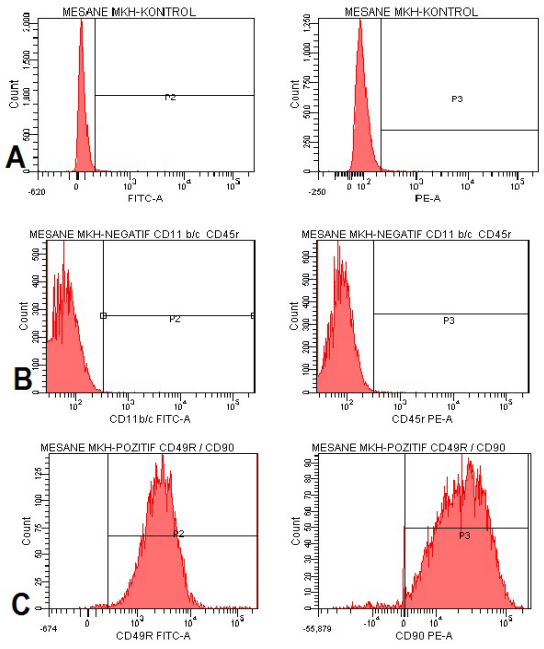

Figure 2. Stem cells definations in flow cytometry devices
A. FITC-A and PE-A negative staining (control)
B. CD 11 a / b (-), CD 45R (-) markers were negative
C. CD 49R (+), CD 90 (+) markers were positive

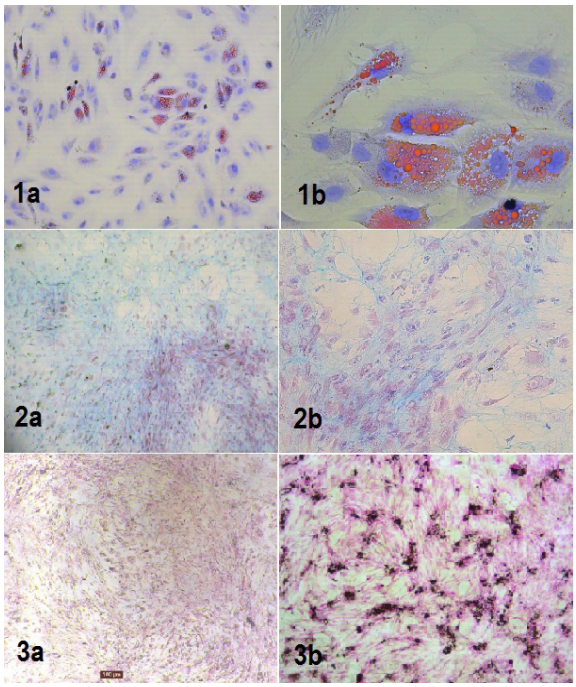

Figure 3. Cells differentiation into adipocyte, chondrocyte and osteocyte cells through differentiation methods.
3.1. Differentiation into adipocyte cells (.Oil-red x 4 (1a) and x40 (1b) magnifications),
3.2. Differentiation into chondrocyte cells (Alcian blue x4 (2a) and x40 (2b) magnifications),
3.3. Differentiation into osteocyte cells (Von kossa x4 (3a) and x40 (3b) magnifications).

Table 1. The weight and biochemical values of the groups

Variables	NR-PBS	DM-PBS	DM-IP	DM-D	*P**
Plazma GluC (mg/dL)	142 ± 29.3	452 ± 74.5	511.5 ± 82.6	520.3 ±44.8	< 0.001
LDL (mg/dL)	11.3 ± 4.9	11.6 ± 2.3	13.6 ± 2.4	10.2 ± 1.5	0.28
HDL (mg/dL)	30.2 ± 4.3	28.8 ± 3.5	27.7 ± 2.9	31.6 ± 3.0	0.24
TAG (mg/dL)	95.3 ± 17.0	109.0 ± 17.5	106.8 ± 25.2	91.7 ± 13.8	0.67
Body Weight (mg)	511±26.2	323.3±31.3	320.5±19.6	322.1±23.1	< 0.001
Bladder Weight (mg)	0.23±0.04	2.12±0.13	0.77±0.26	0.58±0.9	< 0.001

Abbrevations: GluC: glucose, TAG: Triacylglyceride, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein
One way analysis of variance (ANOVA) was used for the difference between groups
* $P < .05$ was considered statistically significant.

light and dark, unlimited water source and food and also the required heat and moisture. Diabetes was induced by a single dose 45 mg/kg Streptozocin-STZ (Sigma-Aldich) via intraperitoneal injection (IP) in 18 rats after excluding 6 rats as the control group. Body weight and blood glucose measurement was done every week regularly to prevent sudden blood sugar and body weight drops or increases.

Blood glucose values 300 mg/dL and over were accepted as diabetic.[9] Diabetes was observed in all STZ-applied rats. All rats including the control group were opened through laparotomy for the application of the treatment protocol, their bladders were exposed and injections were applied in their bladder walls 12 weeks after STZ application. A single dose injection was applied at the same location for all rats into the bladder dome using a 1 cc insulin syringe via laparotomy as described before.[10]

Stem cells were transferred via IP (DM-IP group, n:6) and direct injection to the bladder (DM-D group, n:6) and compared to Phosphate Buffered Saline (PBS) injected diabetic rats (DM-PBS group, n:6) and age-matched non-diabetic groups in which Phosphate Buffered Saline (PBS) was injected into the bladder (NR-PBS group, n:6).

All rats were kept in separate cages after the surgical operation and normal care was continued.

A cystostomy catheter was surgically placed in all rats for urodynamic evaluation four weeks after PBS and stem cell injections (a total of 16 weeks after STZ application). The catheter was tunneled subcutaneously and then was brought out from the rear neck of the rat through a nearly 0.5cm incision place before starting. Injector end and vein cannula were located for providing SF infusion through the catheter for urodynamic studies and were fixed to the skin with 2/0 silk.

Urodynamic measurements were made at least 72 hours after the catheter was placed and all urodynamic measurements were completed in 48 hours. Then the bladders of the rats were extracted for histological examination. The experiment was completed in a total of 24 weeks. The schematic representation of the experimental procedure is summarized in **Figure 1**.

Acquisition of Mesenchymal Stem Cells

Bladder of a neonatal male Sprague-Dawley rat was extracted under deep ketamine and xylazine anesthesia[11] and was placed in Dulbecco's Modified Eagle Medium(DMEM) (Lonza, Belgium). The material was placed in T25 flasks with an explant method. It was kept in 5% CO2 incubator for 15-20 minutes. 20% Fetal bovine serum (Lonza, Belgium), 2% L-Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin, Amphotericin (Biological Industries, Israel) and 77% DMEM were added. The medium was changed every three days. The improvement of the cells was observed with a Leica invert microscope (Leica, Germany). Considering the improvement of the cells 15 days later, the passage operation was made. At the end of the second passage, the cells were assessed in a flow cytometry device (FACS AriaIII, USA) testing for CD11b/c (BD, USA), CD45 (BD, USA), CD49 (BD, USA), CD90 (BD, USA) surface markers (**Figure 2**).

The cells were differentiated into adipocyte, osteocyte, and chondrocyte cells through differentiation methods. Both the number and vitality of the cells were checked on the Countess® Automated Cell Counter (Invitrogen, USA) device (**Figure 3**).

We used 5-Bromo-2'-deoxyuridine (BrdU) which is a thymidine analog used to identify proliferating cells for the tracking of transferred nMSC-Bs. Cells were stained with BrdU after the second passage and injection were made in the bladder wall with PBS in 300 microliters so that the resultant BrdU-stained cells would be 2.10^{6} [12].

Evaluation of Urination Functions

Urination functions of the groups were evaluated with conscious cystometry according to the procedure described previously.[13] To be able to acquire healthy cystometry records, rats were taken into metabolic cage nearly 30 minutes before the recording and their adaptation was ensured for the cystometry. The infusion rate was 10 mL/ hour, the temperature of the physiologic saline was 37 °C in order to avoid bladder overactivity provocation by instilling cold solution. The animals

Table 2. The urodynamic study parameters of the groups

Variables	NR-PBS	DM-PBS	DM-IP	DM-D	*P**
MVP (mmHg)	21.54 ± 1	13.27±0.78	16.27 ± 0.61	28.59 ± 2.09 < 0.001	< 0.001
UI (minute)	4.22 ± 0.71	16.8 ± 4.56	13.2 ± 1.85	9.01 ± 1.52 < 0.001	< 0.001
UV (mL)	0.40 ± 0.07	1.49 ± 0.43	1.13 ± 0.11	0.82 ± 0.10 < 0.001	< 0.001
RV (mL)	0.02 ± 0.01	0.18 ± 0.08	0.15 ± 0.10	0.08 ± 10.07 0.006	0.006

Abbrevations: MVP: maximum voiding pressure, MI: urination micturition, UV: urine volume (UV), RV: residual volume
One way analysis of variance (ANOVA) was used for the difference between groups
* P < .05 was considered statistically significant.

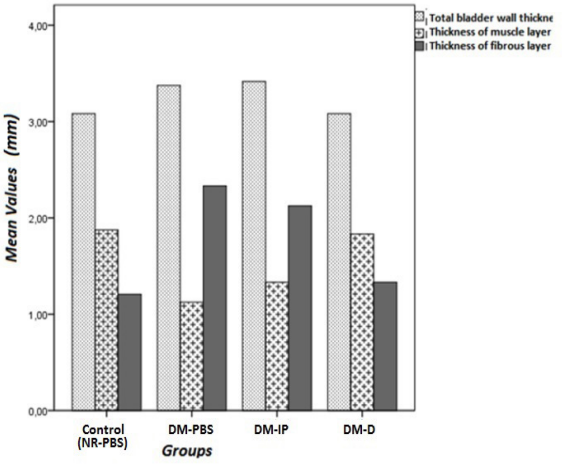

Figure 4. Graphical representation of smooth muscle, fibrosis and total bladder wall thickness.
Smooth muscle layer thickness was significantly lower in DM-PBS and DM-IP groups compared to NR-PBS and DM-D groups ($P < .001$). No statistical difference was observed in smooth muscle layer thickness among DM-D and control group ($P = .99$). No statistical difference was observed in fibrosis tissue thickness measurement results among DM-D and NR-PBS groups ($P = .9$)

were observed in real-time in order to avoid complications such as twisting or kinging of the catheter since the animal was freely moving. Maximum voiding pressure (MVP), urination interval (UI), urine volume (UV), and residual volume (RV) were evaluated in conscious cystometry over a period of 60 minutes for all rats.

Measurement of Biochemical Parameters

Euthanasia was carried out under deep ketamine and xylazine anesthesia to the rats after cystometric examinations and histopathological samples taken surgically. Blood samples taken from vena cava were centrifuged for biochemical examination and the serum and plasma separated. Glucose values (GluC), Blood Urea Nitrogen (BUN), Creatinine (Cr), Triacylglyceride (TAG), High-Density Lipoprotein (HDL) and Low/Very Low-Density Lipoprotein (LDL/VLDL) were analyzed from blood samples taken.

Evaluation of Histopathological Samples

The bladders of rats were surgically extracted. Dissected bladder tissue samples were placed in buffered formalin for 24 hours and were taken through follow-up stages. They were kept in 70% - 80%-90%-96 and absolute alcohols for 1-2 hours during follow-up. They were exposed to xylene to become transparent in two stages lasting an hour and also in paraffin at 56°C for two hours in order to harden the tissues in the final stage.
Bladder tissue samples were taken as 3-4 µm thick cross-sections with microtome device buried in paraffin blocks. The cross-sections taken were opened in a hot water bath at 37 °C, placed on a slide and exposed to deparaffinization operations consisting of two stages of xylene application each lasting 1 hour and 10 minutes in an incubator at 60°C. Each was exposed to absolute alcohol, 96%, 90% and 70% alcohol in order, each lasting a minute for hydration. Bladder tissue samples on slides were stained with Hematoxylin, and eosin (H&E), Masson's trichrome, and smooth muscle actin (SMA). The

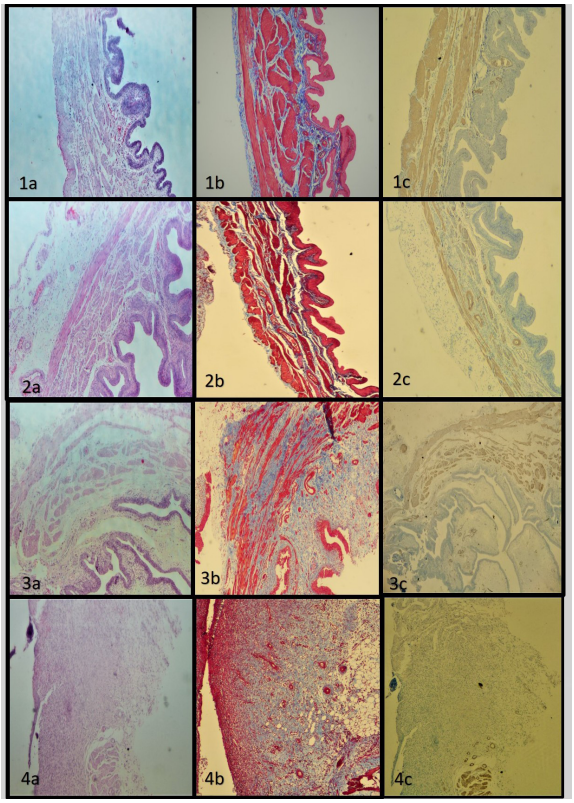

Figure 5. Immunohistochemical staining images of the groups.
5.1a. Normal rat bladder covered with intact epithelium and normal thickness of muscle tissue and thin serosa (H&E x40).
5.1b. Normal muscle tissue without fibrosis (Masson's trichrome x40).
5.1c. Muscular tissue with normal thickness was immun-positive stained with immunohistochemical stain smooth muscle actin (SMA x40).
5.2a. DM-D group shows a slight decrease in muscle mass with increased edema and thickness of serosa (H & Ex40).
5.2b. There was mild muscle atrophy in the DM-D group. (Masson's trichrome x40)
5.2c. Mild muscle atrophy in the DM-D group was specifically monitored by immunohistochemical SMA. (SMAx40)
5.3a. Submucosal edema and more pronounced muscle tissue loss in the DM-IP group (H & Ex40)
5.3b. DM-IP group showed muscle mass loss and fibrosis between the muscle tissues shown by Masson's trichrome (Masson's trichrome x40).
5.3c. Compact structure loss of muscle tissue was observed in DM-IP group (SMAx40)
5.4a. Complete layer loss and fibrosis development of muscle tissue in rat bladder in DM-PBS group (H & Ex40)
5.4b. Fibrosis in the DM-PBS group (Masson's trichrome x40)
5.4c. Full-thickness muscle loss with SMA in the DM-PBS group (SMAx40)

total bladder wall, smooth muscle (SM) and fibrosis thickness were evaluated under an Olympus BX51 light microscope (ToupTek Photonics, P.R.China) by a pathologist who was blinded to treatment.

Statistical Analysis

Statistical analysis was performed with SPSS, v.20.0 statistical software (SPSS, Inc., Chicago, IL, USA). Shapiro-Wilk's test was used to test whether the variables had a normal distribution. $P > .05$ was evaluated as normal distribution. One way analysis of variance

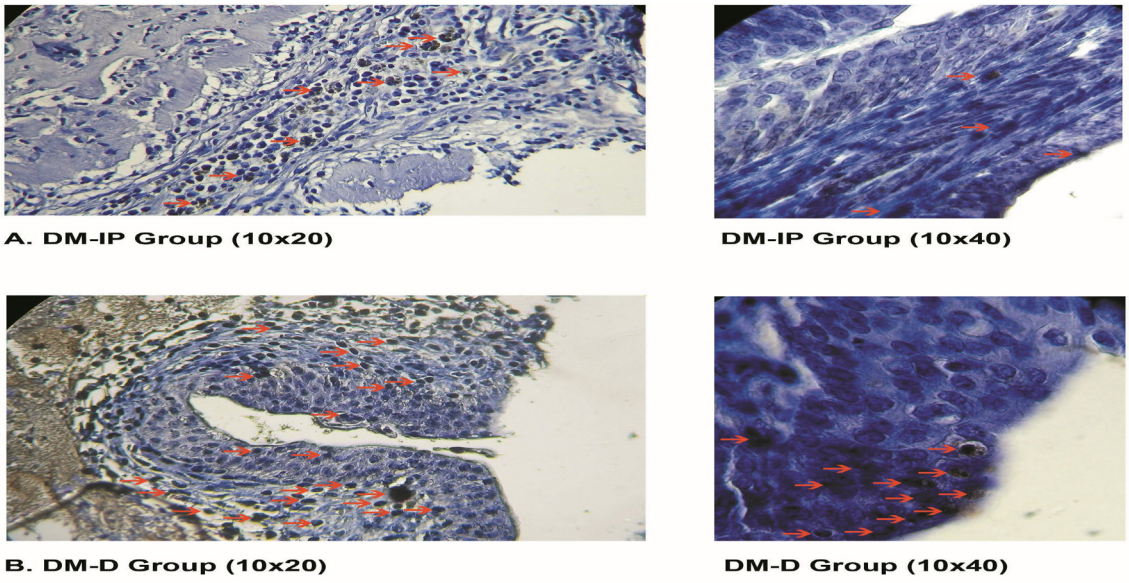

Figure 6. Tracking of BrdU-labeled nMSC-B in the bladder.
A: In the DM-IP group, BrdU-positive cells were located in submucosal and muscular layers shown in 10x20 and 10x40 magnifications
B: In the DM-D group, BrdU-positive cells were located mainly widespread in submucosal and muscular layers and also a few in mucosal layer, too shown in 10x20 and 10x40 magnifications.

(ANOVA) was used for the difference between groups in variances with a normal distribution. (Post Hoc) Tukey analysis was used for paired comparison. $P < .05$ was considered statistically significant.

RESULTS

Metabolic and Biochemical Results

While there was no statistical difference between diabetic groups in the average body weights, they were significantly low in all three diabetic groups compared to the control group ($P < .001$). The bladder weights of diabetic groups were higher than the control group ($P < .001$). While there was no difference between the diabetic groups in glucose values, they were significantly higher in all diabetic groups compared to the control. A statistical difference was not observed between all groups in the other biochemical parameters studied (**Table 1**).

Histopathological Findings and Tracking of BrdU-Positive Cells

No significant difference was observed between the groups in total bladder wall thickness values ($P = .327$), but smooth muscle layer and fibrosis tissue thickness were statistically significant between the groups in one way ANOVA test ($P < .001$). DM-PBS group showed decreased SM, increased fibrosis compared to NR-PBS ($P < .001$ for both parameters). Stem cells improved the bladder structure in DM-IP and DM-D groups. Although, the improvement was not statistically significant in SM and fibrosis in the DM-IP group compared to DM-PBS group ($P = .522$, $P = .680$), it was statistically significant in DM-D group in which stem cells were transferred directly to the bladder ($P < .05$). No statistical difference was observed in the SM and fibrosis between DM-D and NR-PBS groups ($P = .99$, $P = .90$). Graphical representation of smooth muscle, fibrosis and total bladder wall thickness showed in **Figure 4**.

The desquamation of mucosa, loss of muscle tissue and increased fibrosis in diabetic groups have been shown with different staining techniques such as H&E, Masson's trichrome, and SMA. Stem cells improved the bladder structure in DM-IP, and DM-D groups. The improvement was more significant in the DM-D group in which stem cells were transferred directly to the bladder (**Figure 5**).

A small number of BrdU-positive nMSC-Bs were observed in mucosal and submucosal layers in the DM-IP group, whereas BrdU-positive nMSC-Bs were observed as widespread in the mucosal, submucosal and muscular layers in DM-D group (**Figure 6**).

Assessment of voiding function

MVP, UI, UV and RV amounts were compared using conscious cystometry (**Figure 7**). Contractions were 2-5, 3-5, 5-7 and 11-18 within 60 minutes in DM-PBS, DM-IP, DM-D and N-PBS groups, respectively. The MVP values in the DM-PBS group were lower compared to DM-IP, DM-D, and N-PBS groups (13.27 ± 0.78 vs 16.27 ± 0.61, 28.59 ± 2.09, 21.54 ± 1.00, respectively). The MVP showed significant differences between the groups in one way ANOVA and post hoc paired comparison analysis ($P < .05$). The voiding function variables showed in **Table 2**.

The UI, UV and RV showed differences between the groups in one way ANOVA analysis ($P < .05$). The UI of the diabetic groups were statistically higher compared to the control in the post hoc analysis ($P < .05$). While there was no statistically significant difference between the DM-PBS and DM-IP groups in the UI ($P = .110$), it was significantly shorter in the DM-D group than the DM-PBS group ($P < .001$).

The UV of the diabetic groups were statistically higher compared to the control in the post hoc analysis ($P < .05$). While there was no statistically significant difference between the DM-PBS and DM-IP groups in the

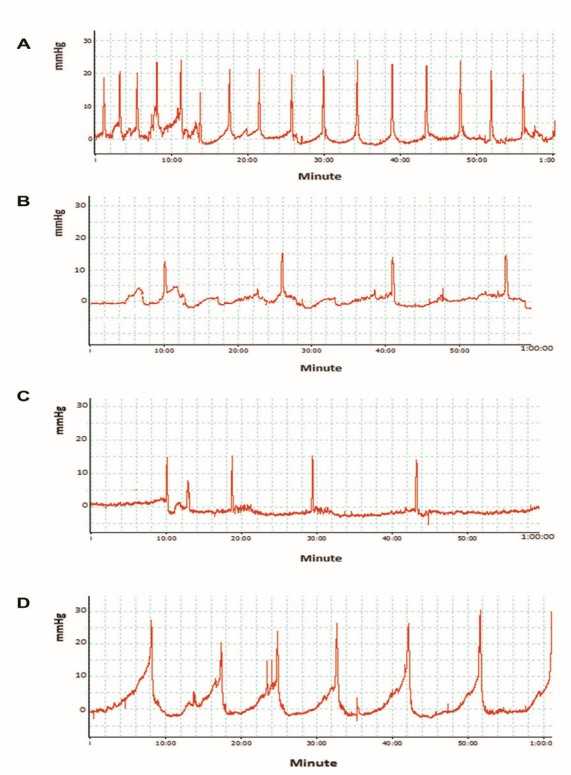

Figure 7. Conscious cystometry images of groups: **A:** NR- PBS, **B:** DM-PBS, **C:** DM-IP, **D:** DM-D.
The figure represents the conscious cystometry images of the groups. Maximum voiding pressure (MVP), urination interval (UI), urine volume (UV) and residual volume (RV) were evaluated in Conscious cystometry over a period of 60 minutes for all rats. Contractions were 2-5, 3-5, 5-7 and 11-18 within 60 minute in DM-PBS, DM-IP, DM-D and NR-PBS groups, respectively. The MVP showed significantly differences between the groups in one way ANOVA and post hoc paired comparison analysis ($P < .05$). The UI, UV and RV showed differences between the groups in one way ANOVA analysis ($P < .05$). The UI,UV of the diabetic groups were statistically higher compared to the control in the post hoc analysis ($P < .05$).The RV was statistically higher in the DM-PBS and DM-IP groups compared to the control group in the post hoc paired comparison analysis ($P = .008$, $P = .026$) while there was no statistical difference between control and DM-D ($P = .526$).

UV ($P = .06$), it was significantly shorter in the DM-D group than the DM-PBS group ($P < .001$)
The RV was statistically higher in the DM-PBS and DM-IP groups compared to the control group in the post hoc paired comparison analysis ($P = .008$, $P = .026$) while there was no statistical difference between control and DM-D ($P = .526$). No statistically significant difference was observed between the diabetic groups in residual volume ($P > .05$).
One way ANOVA test showed statistical differences between the groups in all parameters ($P < .05$). Although improvement was observed in all parameters, no statistical difference was observed comparison of UI, UV, and RV parameters between DM-IP and DM-PBS in the post-hoc analysis ($P = .110$, $P = .061$, $P = .945$). Only MVP values were statistically significant between DM-IP and DM-PBS ($P < .05$).
Post-hoc analysis showed significant improvement in MVP, UI and UV parameters between DM-D, and DM-PBS groups ($P < .05$ for all parameters), but no statistically significant difference was observed in the RV values ($P = .135$).

DISCUSSION

Even though DBD is not a life-threatening disease, it significantly affects the quality of life. The combination of both storage and voiding symptoms coexist in DBD. It has been questioned whether these two different symptom groups are present in DBD or whether these symptoms may change due to changes in bladder structure over time in the natural process of diabetes. Daneshgari et al. revealed that diabetic bladders may undergo a transition from the compensatory to a decompensated phase depending on time and transition in the streptozocin rat model and this may begin 9 to 12 weeks after induction.[9] Our urodynamic results were decreased MVP, increased UV, RV and UI in the non-stem cell treated diabetic group. These findings are characterized by signs of end-stage DBD (hypocontractile or underactive bladder) in accordance with this study.
Cellular based animal studies have shown promising results in DBD. Stem cells have three main features. These are self-renewable, differentiation to different cell types (differentiation), and easy replication. Several different types of stem cells such as adipose-derived stem cells, bone marrow stem cells (BMSCs), skeletal muscle-derived stem cells, muscle precursor cells,[14] Urine-derived stem cells (USCs)[15] and recently human amniotic fluid stem cells (hAFSCs)[16] are used to treat bladder dysfunction in animal models.
Although the mechanism of action of stem cell therapies in bladder dysfunction is not fully known, they show effects mainly in migration, differentiation, and paracrine effect. The migration of mesenchymal stem cells to damaged sites has been shown in many stem cell researches to date. Human studies have also shown the release of chemokine, such as SDF-1 in damaged sites.[17]
A few stem cells are transformed into bladder smooth muscle, although one of the main mechanisms of action was differentiation. Bone marrow (BM), BM-MSC, muscle, ADSCs, USCs are shown to be transformed into bladder smooth muscle cells[14,18] and demonstrated their contractile activity after seeding.[19]
In this study, nMSC-B cells were marked with BrdU before transfer, and the tissue samples taken after treatment were shown to migrate to the damaged bladder. A small number of BrdU-positive nMSC-Bs were observed in mucosal and submucosal layers in the DM-IP group, whereas BrdU-positive nMSC-Bs were observed as widespread in the mucosal, submucosal and muscular layers in the DM-D group. In a similar study Zhang et al.[20] showed EbU-positive ADSCs in muscle and submucosal layers in Type2 diabetic rats. In our study, unlike this study, BrdU-positive nMSC-Bs were observed as widespread in the mucosal layer in addition to the submucosa in DM-D group.
Liu et al. showed that ADSCs express urothelial markers when cultured with urothelial cells which have a very specialized structure and functions.[21] It has been reported that this urothelial differentiation results from the direct cellular contact of ADSCs with the existing urothelial cells when co-cultured, namely the paracrine effect. In our study, we believe that the BrdU labeled

cells observed in the mucosa in the DM-D group are caused by a similar effect.

Our results show that there was an improvement in MVP values in stem cell-treated groups. This improvement was more evident in the DM-D group. Most recent studies emphasize the importance of the paracrine effect of stem cells by direct application to the target organ and MSCs show their main functional properties through the paracrine effect.

Molecules such as Vascular Endothelial Growth Factor (VEGF), nerve growth factor (NGF), Insulin-Like Growth Factor-1 (IGF-1), Hepatocyte Growth Factor (HGF), and basic Fibroblast Growth Factor (bFGF) released from the damaged site locally, stimulate tissue recovery at many potential levels, affect the cellular activity and provide tissue regeneration, and neuroprotectivity in the damaged sites via their paracrine function.[14,22]

In the current study, we think that the functional results may have been due to the more prominent improvement in the paracrine effect of stem cells leading to a restoration of the bladder functioning mechanism in the DM-D group. We observed that the MVP values of DM-D were also higher than the control. This may be due to the up-regulation of cytokines such as NGF which released by stem cells in the acute period. We think that this high contractile response will return to normal values after the washout of the cytokines over time. Lockhart et al. showed the acute modulatory effect of NGF and that the concentration of NGF in the growth medium affects the level of synaptic transmission in cultures of sympathetic neurons and cardiac myocytes.[23]

In similar study to ours with a similar study design, using Type 2 diabetic rat model Zhang et al. showed that the direct application of ADSCs to the bladder produced more effective results than via tail vein.[20] Although they did not have a successful response in all rats with stem cells transplanted directly to the bladder, we obtained similar results in all rats in the DM-D group in our study. This was caused by the difficulty of stem cell application in the bladder, and we have observed that stem cells may go into the bladder space instead of the bladder wall when injected carelessly into the very thin rat bladder.

In the present study, immunohistochemical examination results revealed that the DM-PBS group showed decreased bladder smooth muscle, increased fibrosis, and desquamation in urothelial compared to NR-PBS group ($P < .001$). Stem cells improved the bladder structure in DM-IP and DM-D groups. The improvement was more significant in the DM-D group. No statistical difference was observed in the SM and fibrosis tissue thickness measurement results among DM-D and NR-PBS groups ($P = .9$) (**Figure 5**).

Many researchers in this area have reported that the underactive bladder (UAB) has multifactorial pathophysiology involving detrusor, urothelial, urethral, and autonomic nerve disorders. Polyuria and hyperglycemia independently contribute to the pathogenesis of DBD. While polyuria causes significant bladder hypertrophy in the early stage of diabetes, oxidative stress (OS) plays an important role in late phase dysfunction of the bladder caused by chronic hyperglycemia.[3,24]

Oxidative damage induces apoptosis leading to bladder smooth muscle damage and contributes to diabetic cystopathy.[25] Liang et al. showed that the expression of 8OHdG which is free radical-induced oxidative DNA damaged products increased significantly in the diabetic bladder at weeks 4 and 12 after DM induction. However, after transplantation of hAFSCs, into the bladder, 8OHdG immunoreactivity reduced.[16] The histopathological findings obtained in our study may have been due to this mechanism.

On the other hand, the growth factors mentioned above play an essential part in the reduction of fibrosis in injured organs, which implies that paracrine effects play a more crucial role in the restoration of the injured organ by reducing fibrosis rather than by cell incorporation.[14,20]

Until now, few articles have reported the effects of stem cells in improving diabetic bladder dysfunction[14]. Our study is the first to use nMSC-B for the treatment of diabetic bladder dysfunction. The nMSC-B can be obtained from the bladder of neonates, grow easily in culture, and appear phenotypically stable, these stem cells may act as a novel source for cell transplantation therapy for DBD.

MSCs, are a group of adult SCs that can be harvested from almost every tissue of the body, and these are very similar in their biological and functional properties, regardless of their tissue of origin[26,27].

MSCs with fibroblast-like cells are self-renewing cells with the pluripotent property of differentiation into various cell types, including osteoblasts, chondrocytes, myocytes, adipocytes, and neurons[28]. In our study cells were differentiated into adipocyte, osteocyte, and chondrocyte cells through differentiation methods. Both the number and vitality of the cells were checked on the Countess® Automated Cell Counter (Invitrogen, USA) device (**Figure 3**).

MSCs are also defined by expressing some surface markers. Sca-1, CD105, CD73, CD29 and CD90 positive, CD31, CD34, CD45 and CD11b are negative markers. These surface markers are also used to obtain MSCs (29). In the present study, we defined the cells CD11b/c (BD, USA), CD45 (BD, USA) as negative and CD49 (BD, USA), CD90 (BD, USA) as positive surface markers in flow cytometry device (FACS AriaIII, USA) at the end of the second passage of extracted neonatal bladder (**Figure 2**).

A difference between our study and others is that overactive models that are seen in the compensatory period of DBD have been used in most of the studies done so far, but we have used the UAB model, which is seen in the decompensated period of diabetes. We think that it is important that nMSC-B provided improvement in the decompensated DBD.

The most important difference of this study from the others is that, the MSC was taken from the same organ as allogenic and was taken from the newborn bladder which is richer in the number of stem cells. The investigations show that fetal MSCs differ not only numerically but also in the development of tissue from adult stem cells, in cell division and in genes involved in immunologic antigen presentation[6-8]. The types of stem cells obtained from allogeneic organs are thought to be more beneficial when used for damage that occurs in the same organ[30]. We believe that stem cell transplantation from a neonatal bladder that has been lost for any reason will have a high success rate because it is both allogeneic and has the advantages of neonatal MSCs.

There are some limitations of this study; first, we didn't show the effect of stem cells on molecular changes such as muscarinic receptors, NGF, OS factors, IGF-1,

VEGF in DBD. Second, we did not directly examine cellular differentiation after nMSC-B transplantation into the bladder. Third, long-term studies are required for late period efficacy of nMSCB treatment.

CONCLUSIONS

In this study, we have shown that the nMSC-B provided amelioration in DBD. We think that MSCs can be obtained from the aborted bladder of neonates who die for any reason during or after birth. It is a new MSC origin that can be applied in the treatment of DBD. We also consider the application of stem cells directly on the bladder wall to be more effective than IP administration. Further experimental studies are needed to investigate the mechanisms of action of transplanted nMSC-B in how they improve DBD and clinical application of this study.

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CONFLICT ON INTEREST

There is no potential conflict of interest relevant to this article.

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