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ORIGINAL ARTICLE

The effect of adipose-derived stem cells on augmentation ileocystoplasty: A pilot study

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KEYWORDS

Derived stem cells;
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5-ethynyl-2-deoxyuridine

ABBREVIATIONS

AI, augmentation ileocystoplasty; ADSC, adipose-derived stem cell; EdU, 5-ethynyl-2-deoxyuridine; RU, renal ultrasonography; α-SMA, α-smooth muscle actin; DAPI, 4′,6-diamidino-2-phenylindole; RECA, rat endothelial cell antibody

Abstract *Objectives:* Incorporation of intestinal tissue into urinary tract elicits many metabolic and mechanical complications due to anatomical and physiological differences. Adipose-derived stem cells (ADSCs) improve vascularity and functional outcomes by a paracrine mechanism. In a pilot study we investigated whether ADSCs can survive in the augmented bladder and improve its function.
Materials and methods: Autologous ADSCs were harvested from rat paragonadal fat and cultured before injection into a rat model of augmentation ileocystoplasty (study group). Control augmented bladders were injected with cell-free saline. Eight weeks later, rats underwent abdominal ultrasonography for upper tract changes and were examined by conscious cystometry to determine bladder function. After extirpation, augmented bladders were examined using Masson trichrome staining for connective tissue and muscle content, immunohistochemistry for α-smooth muscle actin, and rat endothelial cell antigen staining for endothelial cells. Changes in the extracellular matrix were assessed by determining the elastin content. ADSCs were labelled and tracked by 5-ethynyl-2-deoxyuridine nuclear staining.
Results: Abdominal ultrasonography showed better preservation of upper tract function in the ADSC group than in the saline-treated group ($P = 0.007$). After 2 months there were no differences in the variables assessed by conscious cystometry between the ADSC and saline-treated groups. How-

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ever, the bladder weight was significantly greater in the ADSC-treated group. On immunohistochemistry, the implanted ADSCs survived up to 8 weeks but did not transdifferentiate into smooth muscle or endothelial cells.

Conclusion: These results suggested a potential role of ADSCs in modifying the intestinal segment in augmented bladders; this role has to be further elucidated.

Introduction

Augmentation enterocystoplasty, especially augmentation ileocystoplasty (AI), is a common surgical procedure to treat neurogenic and myogenic bladder. However, the gastrointestinal tract is an imperfect substitute for the urinary tract; particularly, its epithelial semipermeability for non-physiological fluids and electrolytes often leads to metabolic abnormalities, thus increasing demand on the kidneys [1]. In addition, enterocystoplasty also causes chronic urinary retention that can lead to further renal deterioration [1]. Therefore, an important goal of therapy in patients undergoing augmentation enterocystoplasty is the preservation of renal function [1].

Stem cells are well known for their regenerative capacity and have been shown to improve the outcome of various surgical procedures. In particular, bone marrow stem cells have been shown to improve the healing of ischaemic colonic anastomosis [2], and sutures coated with adipose-derived stem cells (ADSCs) have also enhanced the healing of weak colonic anastomoses [3]. In our laboratory we have used ADSCs to treat various urological diseases, such as stress urinary incontinence, overactive bladder and erectile dysfunction [4–8]. We therefore tested whether ADSCs could improve the outcome of AI, e.g. the degree of hydronephrosis. We present our preliminary findings that suggest improved preservation of renal function.

Materials and methods

The study comprised 25 male Sprague–Dawley rats (2 months old; Charles River Laboratories, Wilmington, MA, USA). Their care and treatments were approved by the Institutional Animal Care and Use Committee at our institution. After harvesting their paratesticular fat, the rats were randomly divided into three groups, i.e. sham (five), AI-only (10), and AI + ADSC (10).

Each rat in the AI + ADSC group had adipose tissue harvested for the purpose of ADSC isolation. Under 2% isoflurane anaesthesia, a midline abdominal incision was made to expose the perigonadal fat pad. A specimen of paratesticular fat was harvested and placed in PBS on ice. The wound was then closed in two layers with absorbable suture. After rinsing with PBS, the adipose tissue was minced into small pieces and then incubated in a solution containing 0.075% collagenase type IA (Sigma–Aldrich, St. Louis, MO, USA) for 1 h at 37 °C, with vigorous shaking for 15 s at 20-min intervals. Afterward, the top lipid layer was removed and the remaining liquid portion centrifuged at 1000*g* for 10 min at room temperature. The pellet was then treated with 160 mm NH_4Cl for 10 min to lyse red blood cells. The remaining cells were suspended in 10 mL of Dulbecco's Modified Eagle Medium supplemented with streptomycin, fungizone, penicillin and 10% foetal bovine serum. The suspension was filtered through a 70-μm cell strainer, plated at a density of 10^6 cells in a 10-cm dish, cultured at 37 °C in 5% CO_2, and, 24 h later, rinsed with PBS. After 3–5 days of further incubation, the cells were analysed for cell-surface marker expression and then labelled with the thymidine analogue 5-ethynyl-2-deoxyuridine (EdU; Invitrogen, Carlsbad, CA, USA) for 24 h [4–8] and then harvested; $\approx 3 \times 10^6$ EdU-labelled ADSCs in 1 mL of PBS were used for each autologous injection.

Several different surgical procedures for AI were tested before selecting the one described here. Rats were fasted the night before surgery to reduce the bowel content. Under 2% isoflurane anaesthesia, a midline abdominal incision was made to expose the ileum and urinary bladder. In the sham group no further treatment was done except for closing the wound. In the other two groups the rats were further treated as follows. Briefly, 1 cm of the terminal ileum was isolated and transected on both sides, while leaving the mesentery intact. The intestine was then re-anastomosed, and the isolated ileal segment was anastomosed to the bladder after excising its dome (Fig. 1). Afterward, 1 mL of PBS and ADSCs were injected into the submucosal layer of the ileal segment in the AI-only group and the AI + ADSC group, respectively. The wound was then closed in two layers with absorbable suture.

At 5 weeks after AI all rats underwent renal ultrasonography (RU, under anaesthesia) using a 7.5 MHz ultrasound probe to assess renal anatomy (Siemens Sonoline Versa Pro., Siemens Healthcare USA, Issaquah, WA, USA).

At 6 weeks after AI bladder catheters were implanted 24 h before conscious cystometry; briefly, under 2% isoflurane anaesthesia, the abdomen was opened through a midline incision and a polyethylene catheter (PE-90, Clay-Adams, Becton Dickinson, Parsippany, NJ, USA) was implanted into the bladder through left lateral wall. The catheter was tunnelled subcutaneously and brought out through a skin incision on the rat's back. Enrofloxacin (2.5 mg/kg, intramuscular) was then administered immediately and 12 h later.

Conscious cystometry was then conducted on the above-treated rat 24 h later in a custom-made tunnel within a metabolic cage (Braintree Scientific, Braintree, MA, USA), which allows the recording of micturition time and volumes. Briefly, the bladder catheter was connected via a T-tube to both a pressure transducer (Utah Medical Products, Midvale, UT, USA) and an infusion pump (Harvard Model 22, KD Scientific, Holliston, MA, USA). After calibrating the pressure transducer to zero, the bladder was filled with room-temperature normal saline at 0.1 mL/min, while recording simultaneous pressure on a sensor input module (model SCXI 1121, National Instruments, Austin, TX, USA) connected to a Dell Pentium 4 computer with Laboratory View 6.0 software (National Instruments). All rats were given ≈10 min for the voiding patterns to stabilize, and their micturition was then recorded for 30 min Thereafter, rats were killed and their bladders harvested for histological analysis.

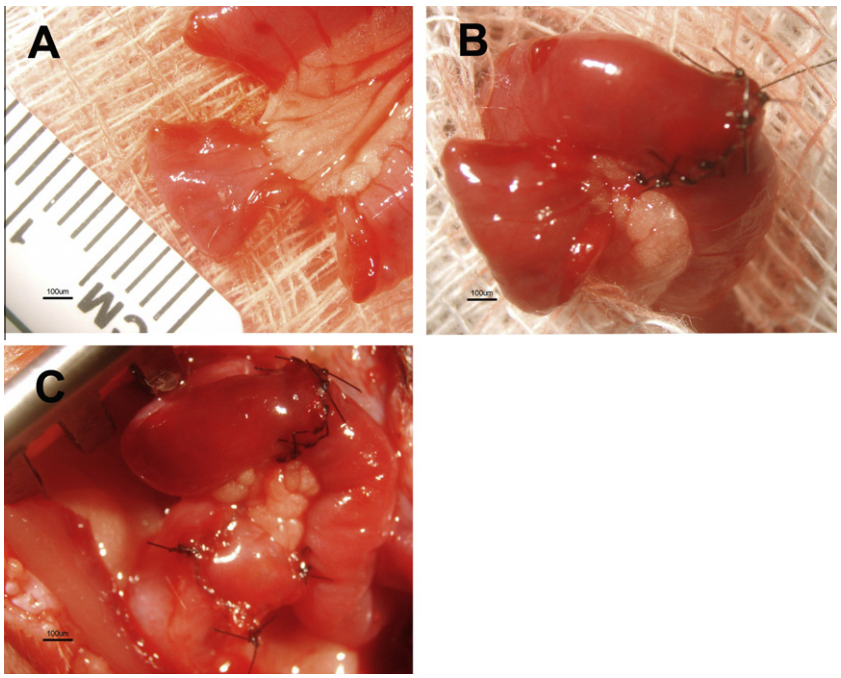

Figure 1 (A) 0.5 cm of terminal ileum is isolated (arrows), (B) re-continuity of ileum is restored, (C) the ileal segment is detubularized and anastomosed to the bladder after its dome has been excised (arrow heads).

The bladders were fixed in cold 2% formaldehyde and 0.002% picric acid in 0.1 m phosphate buffer, pH 8.0, for 4 h, followed by overnight immersion in buffer containing 30% sucrose. The specimens were then embedded in OCT Compound (American Master Tech Scientific, Inc., Lodi, CA, USA) and stored at −80 °C until use. Sections were cut at 5 μm, mounted onto charged slides, and air-dried for 5 min. The slides were stained with Masson trichrome for connective tissue and smooth muscle histology.

EdU staining with immunostaining for α-smooth muscle actin (α-SMA) was done by placing slides in 0.3% H_2O_2/methanol for 10 min. Slides were then twice-washed in PBS for 5 min and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 min at room temperature. After draining this solution from the tissue section, the slides were incubated at 4 °C with anti-α-SMA antibody (Abcam Inc., Cambridge, MA, USA) overnight. After rinsing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After three rinses with PBS, the slides were washed with 3% BSA in PBS twice for 5 min and incubated in 0.5% Triton X-100 in PBS for 20 min at room temperature. The 0.5% Triton X-100 in PBS was removed and each slide was washed twice with 3% BSA in PBS. To each slide was added 0.5 mL Click-iT™ reaction cocktail (Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature in the dark. After rinses with PBS, the slides were incubated with 4′,6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 μg/mL, Sigma–Aldrich, St. Louis, MO). Slides were then washed twice with 1 mL PBS and mounted with 30% glycerol in PBS.

We used rat endothelial cell antibody (RECA) as a marker for endothelial cells to study the changes in vasculature. The slides were rinsed with PBS for 5 min, and treated with 0.3% H_2O_2/methanol for 10 min. After rinsing, sections were washed twice in PBS for 5 min, followed by 30 min of incubation with 3% horse serum in PBS/0.3% Triton X-100 at room temperature. After draining excess fluid, the sections were incubated overnight at 4 °C with mouse anti-RECA-1 (Abcam Inc.). The tissue was immunostained with the avidin–biotin-peroxidase method (Elite ABC, Vector Labs, Burlingame, CA, USA), with 3,3-diaminobenzidine as chromogen, followed by haematoxylin counterstain. The stained tissue was examined with a fluorescence microscope and photographed.

To stain for smooth muscle and collagen, freshly dissected tissue was fixed with cold 2% formaldehyde and 0.2% picric acid in 0.1 m phosphate buffer followed by immersion in buffer containing 30% sucrose. The fixed tissues were then frozen in OCT compound. Sections were cut at 6 μm and immersed in warm (58 °C) Bouin solution for 15 min, rinsed, stained with Weigert Haematoxylin for 10 min, and then rinsed until only nuclei remained stained. Sections were then stained for smooth muscle with Biebrich Scarlet-Acid fuschin for 3 min, rinsed, and immersed in phosphomolybdic acid for 45 min. Next, collagen was stained blue with Aniline Blue for 3 min, and the tissues were rinsed in distilled water for 2 min, immersed in 1% acetic acid for 2 min, and rinsed in distilled water for 2×2 min. Finally, the tissues were rehydrated through increasing concentrations of ethanol, left to air dry, and mounted. To prevent variations in staining, all sections were stained with Masson trichrome at the same time.

For elastin staining, urethral tissue specimens were fixed in cold 2% formaldehyde and 0.002% saturated picric acid in 0.1 μM phosphate buffer (pH 8.0) for 4 h, followed by overnight immersion in a buffer containing 30% sucrose for cryoprotection. The specimens were embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA) cut at 10 μm, mounted on SuperFrost Plus charged slides (Fisher Scientific, Pittsburgh, PA, USA), and air-dried for 5 min The dried tissue sections were immersed in 0.25% potassium permanganate solution for 5 min, cleared in 5% oxalic acid, and soaked in

resorcin-fuchsin solution overnight. After being washed in water, sections were counterstained with Van Gieson solution for 1 min, and then dehydrated in ethanol, cleared in Histo-Clear and mounted with Histomount. To prevent variations in staining, all samples were stained simultaneously using this procedure. For image analysis, five randomly selected fields per tissue per rat for each treatment group were photographed and recorded a still camera and ACT-1 software (Nikon Instruments Inc., Melville, NY, USA).

Data were expressed as the mean (SD) for continuous variables. The continuous data were analysed by one-way anova; if the difference was significant, the Tukey–Kramer method was used to perform pair-wise comparisons. Nominal data such as normal or hydronephrotic kidneys were analysed using the chi-square method; for all tests statistical significance was set at $P < 0.05$.

Results

Two rats died in each group after AI, from bowel obstruction (data not shown). All eight surviving rats in each group were examined at 5 weeks after surgery by RU to detect the presence of hydronephrosis. Each RU was graded as normal or having moderate to marked hydronephrosis (data not shown). The results showed that nine of 16 kidneys in the AI + ADSC group were normal, while two of 16 in the AI-only group were normal ($P = 0.007$); seven and 14, respectively, showed moderate to severe hydronephrosis.

At 6 weeks after AI conscious cystometry showed that one rat in the AI + ADSC group and three in the AI-only group leaked continuously, suggestive of overflow incontinence. When compared with each other or to the sham-treated group, the AI + ADSC and AI-only groups showed no statistical differences in the number of voids or voiding interval, average volume per void, and total volume voided (Table 1). The AI + ADSC and AI-only groups also had no statistical differences in per-void volume or detrusor pressure (Table 1). However, the AI + ADSC group had a significantly larger void volume (Table 1).

The anastomosed ileo-bladder tissue in each AI + ADSC rat was examined at 8 weeks after surgery for the presence of ADSCs, by tracking the EdU label. The results show that many ADSCs congregated in the ileal submucosa (Fig. 2). Double staining for EdU and α-SMA showed no differentiation of ADSC into smooth muscle cells or vascular endothelial cells (Fig. 3A–C). Likewise, double staining for EdU and RECA showed no differentiation of ADSC into endothelial cells.

The anastomosed ileo-bladder tissue in each AI + ADSC and AI-only rat was assessed for muscle vs matrix content at 8 weeks after surgery by trichrome stain (Fig. 4). There was a trend to higher muscle vs. muscle content in the AI + ADSC than in the AI-only group, but the difference was not statistically significant ($P = 0.2$). Nevertheless, the wet weight of the bladder in the AI + ADSC group was significantly higher than in AI-only group (Table 1). The elastin content was also significantly greater in the AI + ADSC than in the AI-only group (Fig. 5).

Discussion

The most serious problem after AI is upper tract deterioration. In this study, RU showed deterioration of the upper tract in 14 renal units in the sham group and seven in the AI + ADSC group; this difference was statistically significant ($P = 0.007$) and signifies better functioning of the augment after ADSC injection.

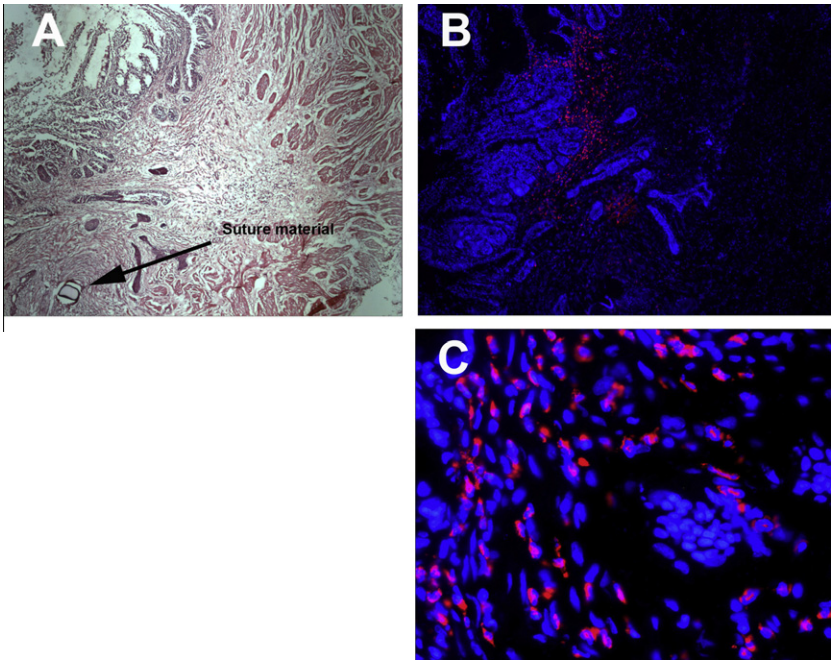

Figure 2 (A) part of the wall of the ileo-augment stained with haematoxylin and eosin. (B) ADSCs labelled with EdU (red stain) are seen overlapping nuclei labelled with DAPI stain and are mainly concentrated into submucosa. (C) At 400× high power, overlapping between red EdU and blue DAPI stains.

Table 1 The results of conscious cystometry.

Variable	AI + ADSC[a]	AI-only[b]	Sham	P^c	P^d
No. of rats	7	4	5		
N voids/30 min	5.85 (0.89)	5.25 (1.25)	6.2 (0.83)	0.3	0.3
No. of rats	7	4	5		
Voiding interval (min)	5.25 (1.02)	5.94 (1.3)	4.9 (0.7)	0.3	0.3
No. of rats	7	4	5		
Average volume/void (mL)	0.65 (0.05)	0.55 (0.25)	0.33 (0.01)	0.02	0.3
No. of rats	7	7	5		
Total volume voided/30 min	3.52 (0.58)	2.82 (0.58)	2.3 (0.14)	0.02	0.03
No. of rats	7	3	5		
Detrusor pressure (cm H_2O)	20.38 (3.8)	21.16 (1.0)	47.3 (2.2)	0.005	0.7
No. of rats	8	8	5		
Bladder weight (mg)	444.7 (45.1)	349.8 (74.9)	197 (9.7)	0.005	0.008

[a] One rat had continuous leaking.
[b] Three rats had continuous leaking.
[c] Differences among all groups analysed by anova.
[d] Differences between AI + ADSC and AI-only groups, analysed by *t*-test.

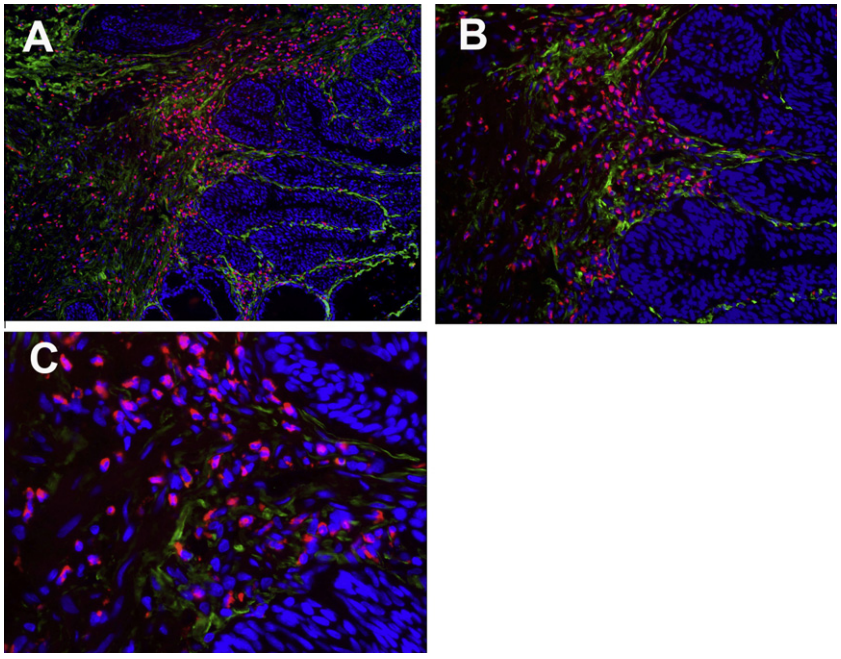

Figure 3 ADSCs are rarely converted to SMA (green stain). A, B, and C are at 100×, 200× and 400×, respectively.

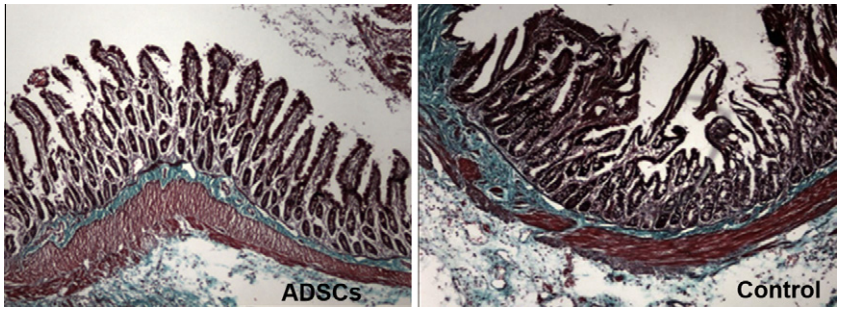

Figure 4 Masson trichrome staining in the AI + ADSC group and AI-only group.

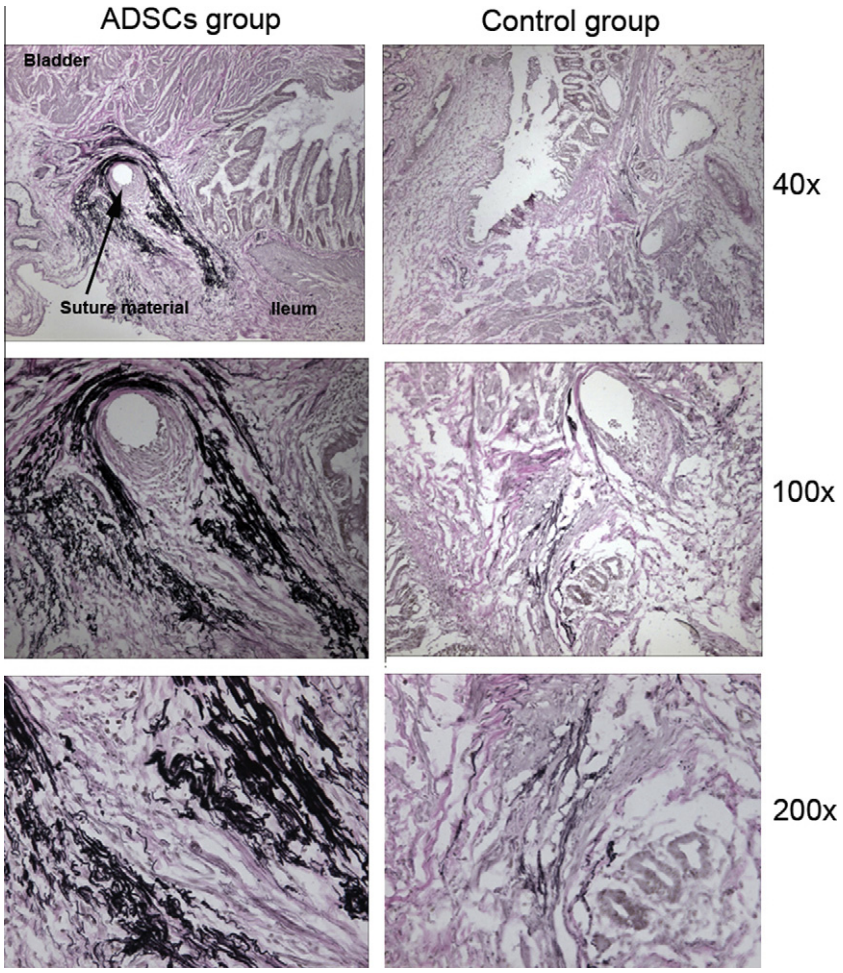

Figure 5 There was more elastin staining in the AI + ADSC group than in the AI-only group.

Conscious cystometry showed that more rats in the AI-only group (three) than in the AI + ADSC group (one) had continuous leakage of infusion fluid. This event suggests overflow incontinence and correlates with upper tract deterioration in these rats. Analysis of the remaining rats showed a better total amount voided per 30 min in AI + ADSC group than in the AI-only group ($P = 0.03$). We do not know the significance of this finding because we did not control the fluid intake of the rats. The wet bladder weight was higher in AI + ADSC group than in the AI-only group ($P = 0.008$). This might be partly due to an increase in muscle content induced by ADSC. Although ADSCs have been reported to transdifferentiate into various cells *in vitro*, we did not detect transdifferentiation of ADSCs into smooth muscle or endothelial cells. This lack of *in vivo* transdifferentiation is consistent with most studies using ADSC and other types of mesenchymal stem cells (reviewed in [9–11]). Nevertheless, the many EdU-positive cells were detected up to 8 weeks suggested that these cells could survive the harsh environment and continuously exert their paracrine effects on the local tissue. We previously showed similar paracrine effects in the urethra, bladder and penis of ADSC-treated animal models [4–8].

The potential role of ADSCs in improving the function of an ileal segment that is in contact with the urinary stream has yet to be elucidated. The present study is considered a preliminary report and is the first to show a potential role of ADSCs in such context. However, more detailed study on a larger population of rats is needed to confirm the findings, as well as elucidate the underlying molecular mechanism. In addition, the effect of ADSC implantation on metabolic sequences of exposing intestinal mucosa to urine, stone formation, production of mucus, malignant transformation, etc. are all important questions that warrant further study.

In conclusion, ADSCs survive up to 8 weeks when injected into an ileal segment of AI in the rat. There were fewer hydronephrotic renal units in the AI + ADSC group than in the AI-only group. Transdifferentiation of ADSC into smooth muscle or endothelial cells was not detected. This suggests a potential paracrine effect of ADSCs on local tissue. Further studies are warranted.

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