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ORIGINAL ARTICLE

The use of mesenchymal stem cells in bladder augmentation

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Abstract
Purpose To compare integration of bladder acellular matrix (BAM) with the bladder when seeded with mesenchymal stem cells (MSC) and when MSC are injected intravenously (IV).
Methods MSCs were isolated from bone marrow of EPM-1 Wistar male rats. Female rats were distributed into: **Group A**—BAM augmentation; **Group B**—BAM augmentation and MSCs IV administered; **Group C**—BAM-MSC seeded augmentation. Animals were killed on postoperative days 7, 14 and 28. Morphological analyses were performed using hematoxylin and eosin and Masson's trichrome, in addition to immunohistochemical staining with α-SMA and neurofilament for assessment of tissue repair. RNAm expression of the SRY gene was used to mark MSCs in the rats killed on postoperative day 28.
Results The muscle layer was best repaired in **Groups B** and **C**. No difference in the repair of the urothelium in the animals in any of the three groups was found. **Group B** presented the smallest inflammatory reaction and the best neural repair on postoperative day 28. None of the animals examined had MSCs in their bladder graft.
Conclusion The MSCs were able to improve repair of the muscle layer and when injected intravenously, they were noted to initiate the neuronal regeneration process.

Keywords Urinary bladder diseases · Tissue engineering · Mesenchymal stem cells

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Introduction

The bladder is an organ of complex structure that can store large volumes, while simultaneously maintaining the pressures low between mictional periods. Injuries resulting from tumors, traumas, infections, inflammatory or neurological diseases affect their functional properties, leading to loss of continence and problems with urine storage, therefore damaging, sometimes in an irreversible way, the renal function [1, 2]. Thus, the great challenge, when one considers replacing the bladder, is to try to maintain or recreate its wall properties.

Surgical interventions using modified gastrointestinal segments are, still today, the main way to increase the bladder capacity and to attempt to improve its compliance. However, those interventions are often time consuming,

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placing the gastrointestinal epithelium in contact with urine, quite often resulting in metabolic and infectious diseases that lead to the formation of stones and that may cause spontaneous perforations of the new reservoir in the medium term, or even malignancies, in the long term [2].

Tissue engineering is currently an alternative to the functional reconstruction of the bladder; however, the culture of urothelial cells remains technically challenging. Those cells are labile and their cultures can be easily contaminated by stromal cells, while muscle cells, of relatively simple culture, may not be available in cases requiring total or partial bladder replacement, such as cloacal or bladder exstrophy, and tumors [3–7]. In such cases, stem cells may come in as an excellent alternative [8]. A stem cell is defined as an undifferentiated cell that, as it divides itself, gives rise to two daughter cells, a similar one (self renovation) and a different one named mother cell committed to a specific strain. Adult stem cells can be found in every tissue; they are considered multipotent (they differentiate only into cells of the same embryonic layer) and are able to maintain the tissues homeostasis and integrity. They remain in a quiescent state in niches and, should an injury occur, they are capable of migrating to the affected site and providing normal cell replacement in this specific tissue. MSCs are a heterogeneous subset of stromal stem cells that can be isolated from many adult tissues and that can differentiate into cells of the mesodermal lineage [9].

MSCs have been seeded in either synthetic or natural biomaterials for the confection of neobladders. Bladder repair appears to take place properly in the initial experiment [10–13]. Nevertheless, systemic administration of MSCs in bladder tissue engineering has not been tested yet.

In this experiment, bladder augmentations were performed using decellularized matrices from heterologous bladders aiming to assess the MSCs activity when directly seeded in those matrices or when intravenously administered.

Materials and methods

Ethical approval

All experimental procedures were approved by the local Research Ethics Committee (CEP no. 0133/10) and conducted in strict conformity with local institutional guidelines and with international standards for manipulation and care of laboratory animals.

MSC isolation and characterization

MSCs were isolated from the tibias and femurs of male Wistar-EPM rats (150–180 g). Flushed cells were subjected to Histopaque (Sigma-Aldrich, St. Louis) density gradient separation, and the mononuclear fraction was harvested. This fraction was cultured on plastic dishes in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10 % fetal calf serum (FCS; Gibco) until the cells reached 70–80 % confluence. Cells were detached with trypsin and cultured until the seventh passage. At the second passage, cells were subjected to osteogenic and adipocyte differentiation assays. For adipocyte differentiation assays, cells were incubated with DMEM low glucose-10 % fetal bovine serum supplemented with dexamethasone (1 μM), isobutylmethylxanthine (0.5 mM), insulin (10 μg/mL), and indomethacin (100 μM) for 3 days, and 1 day incubated with insulin (20 μg/mL) for 21 days. For osteogenic differentiation, MSCs were incubated in DMEM-low glucose and 10 % FCS supplemented with dexamethasone (0.1 μM), ascorbic acid (0.2 mM), and beta glycerol phosphate (10 mM) for 28 days. All reagents were purchased from Sigma-Aldrich. The cells were also phenotyped using the following markers: CD34, CD45, CD90, CD73, CD31, CD29, and CD44 [14]. All antibodies and their respective isotype controls were purchased from BD Biosciences (BD, San Diego).

Preparation of bladder acellular matrices (BAM)

Rabbit bladders were obtained from commercial slaughterhouses and rinsed with PBS. In order to promote cell lysis, isolated bladders were soaked for 14 days in 1 % Triton X-100 (Sigma-Aldrich, St. Louis) and 0.1 % (v/v) ammonium hydroxide. The solution was refreshed every 2 days. The resulting matrix was then washed thoroughly with distilled water for 24 h and cut into 1 cm^2 pieces. After 1 h of UV radiation, the bladder matrix was stored at −20 °C PBS containing antibiotics. To determine if there were any cells remaining, pieces of the bladder matrix were fixed in 10 % formalin overnight, embedded in paraffin wax and stained with hematoxylin and eosin (H&E) and Masson's Trichrome.

Cell seeding and culture in BAM

BAM were thawed, placed on culture slides and exposed to UV radiation under a laminar flow chamber for 1 h. 1×10^6 MSCs from the fourth to seventh passages were seeded onto one side of the BAM. Cells were allowed to adhere for 30 min and then 500 μL DMEM-low glucose and 10 % FCS were gently added. After 24 h, 1.5 mL of medium was added to the dish and the BAM was maintained in culture for 1 week.

MSC treatment

5×10^5 MSCs were intravenously (caudal vein) administered to each female animal on postoperative days 1, 8 and 15.

Experiments

Forty-five female Wistar-EPM rats (150–250 g) were split into three groups: **Group A**—BAM augmentation; **Group B**—BAM augmentation and MSCs IV administered; and **Group C—**BAM-MSC seeded augmentation (Table 1). The animals were anesthetized with an intraperitoneal injection of ketamine and xylasin (150:30 mg/mL). The bladder was exposed through a midline lower abdominal incision and partial cystectomy (1 cm^2 area of dome) was performed. A 1 cm^2 of BAM in **Group A** and **B** or BAM-MSC cell seeded in **Group C** was grafted to the remaining host bladder in a continuous 6.0 absorbable polyglactin suture. The matrix borders were marked with 7.0 polypropylene. The cell-seeded surface was faced to the bladder lumen and the bladder grafts were covered with adjacent peritoneal fat. The abdominal wall and skin were closed in two layers. Animals were killed at days 7, 14 and 28 after grafting.

Histology and immunohistochemistry

The excised bladders were fixed in 10 % buffered formalin overnight and then embedded in paraffin. The 5 μ cut sections were stained with H&E and Masson's Trichrome. In order to assess the evolution of the inflammation and tissue repair, a pathologist examined the sections (4 microscopic fields) in a blind study and assigned a 1–4 score for the following criteria:

(A) Density of neoformed microvessels

- (4) Presence of a large number of neutrophils and macrophages
- (3) Presence of an intermediary number of neutrophils and macrophages
- (2) Presence of a small number of neutrophils and macrophages
- (1) Presence of lymphocytes and plasmocytes.

Table 1 Animals distribution

	Group A BAM	Group B BAM + MSC injection	Group C BAM-MSC seeded
PO 7	3	6	4
PO 14	3	5	3
PO 28	9	6	6
Total	15	17	13

PO postoperative day, *BAM* bladder acellular matrix, *MSC* mesenchymal stem cells

(B) Density of neoformed microvessels

- (4) More than 41 microvessels per large field of magnification
- (3) Between 26 and 40 microvessels per large field of magnification
- (2) Between 16 and 25 microvessels per large field of magnification
- (1) Up to 15 microvessels per large field of magnification.

(C) Density of fibroblasts and myofibroblasts in the implantation area

- (4) Large number of fibroblasts and myofibroblasts
- (3) Intermediary number of fibroblasts and myofibroblasts
- (2) Small number of fibroblasts and myofibroblasts
- (1) Absence of fibroblasts and myofibroblasts.

(D) Density of collagen fibers

- (4) Major density of collagen fibers and smaller cell density
- (3) Intermediary density of collagen fibers as well as intermediary cell density
- (2) Small density of collagen fibers and major cell density
- (1) Absence of collagen fibers.

Immunohistochemistry was performed for smooth muscle anti-alpha-actin—α-SMA (1:200, Clone 1A4, Dako, Glostrup, Denmark) and anti-neurofilament antibodies (1:50, Clone 2F11, Dako). Slides were briefly deparaffinized, rehydrated, and subjected to Tris–EDTA, pH 9, antigen retrieval solution at 95 °C. The endogenous peroxidase activity was blocked with 3 % hydrogen peroxide, and the sections were blocked with protein block solution (Dako). Then, the slides were incubated with either a primary antibody or a negative control reagent, followed by incubation with the labeled polymer (Envision; Dako) using two sequential 30-min incubations at room temperature. Staining was completed by performing a 1- to 3-min incubation with (3.3) tetrahydrocloride and diaminobenzidine substrate chromogen (DAB), which results in a brown-colored precipitate at the antigen site. Hematoxylin counterstaining was performed. Filaments were counted in four microscopic fields of 400× magnification and the values were thus expressed:(4) over five nerve fillets per field; (3) sections with three or four nerve filaments per field; (2) sections with two nerve filaments per field; (1) sections with one nerve filament per field.

For statistical analysis, values from 0 to 2 were used to correlate the neurofilament protein variables and the presence or absence of the entire muscle layer architecture. Such assessment, performed only for the animals killed on

postoperative day 28, considered the following findings: (0) absence of muscle layer architecture; (1) presence of muscle layer architecture with scarce neural network (score 0–2); (2) presence of muscle layer architecture with adequate neural network (scores 3 and 4).

Real-time PCR

Bladder samples from female rats killed on day 28 after grafting were rapidly frozen in liquid nitrogen. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA), and RNA concentrations were determined by Nanodrop (Thermo, Whatham, MA, USA). First-strand cDNAs were synthesized using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Reverse-transcription polymerase chain reaction (PCR) was performed using Syber Buffer (Applied BioSystems, Foster City, CA) and SRY primers (SRY forward: 5′ ATT TAT GGT GTG GTC CCG TGG AGA 3′; SRY reverse: 5′ TTG AGG CAA CTT CAC GCT GCA AAG 3′); hypoxanthine phosphoribosyltransferase (HPRT) forward: 5′ CTC ATG GAC TGA TTA TGG ACA GGA C 3′ and HPRT reverse: 5′ GCA GGT CAG CAA AGA ACT TAT AGC C 3′). Analyses were performed with Sequence Detection Software 1.9 (Applied Biosystems) (SDS). Amplification conditions for Syber primers were 95 °C for 10 min (1 cycle), 95 °C for 10 s, and 60 °C for 2 min (40 cycles). Amplicons were subjected to a 2 % ethidium bromide-stained agarose gel.

Statistical analyses

Statistical analyses were performed in Microsoft Excel, using add-ins for the non-parametric tests and boxplots [15], and Bioestat. Differences between groups were evaluated by non-parametric Mann–Whitney (MW) or Kruskal–Wallis (KW) tests. p values <0.05 were considered statistically significant.

Results

MSC characterization

MSCs were characterized by FACS, labeling positively for CD73, CD90, CD44, and negative for CD34, CD45 and CD31. In addition, they were also differentiated into adipocyte and osteocyte (data not shown).

Histology and immunohistochemistry

Histological examination demonstrated BAMs as being completely acellular. All animals showed early epithelial restitution; most, however, with continuity solution and active borders after 7 days. The adjacent stroma was disorganized, swollen and with a dense and predominantly neutrophilic inflammatory infiltrate. There were neoformed capillaries with exuberant endothelium.

The epithelium had been repaired after 14 days in all groups. The adjacent stroma had fusiform cells in a variable amount, arranged in bundles running parallel to the epithelium stratum and intermingled with a moderate amount of capillary vessels (Figs. 1, 2).

All animals had few cells and a small number of capillary vessels with plain endothelium lining after 28 days. The stroma already had collagenous fibers. In **Groups B** and **C,** however, the muscular tissue was partially organized which could be better observed when stained with α-SMA (Fig. 3).

Thus, the tissue repair in all groups was similar on postoperative days 7 and 14. However, the bladder graft appearance in **Groups B** and **C** resembled that of the native bladder tissue after 28 days.

Significant reduction in the inflammatory process was noted over the time in **Group B** (KW $p = 0.003$). **Group B** presented less inflammation (KW $p = 0.011$) after 28 days. No significant reduction of the inflammatory process was found over the time in **Groups A** and **C** (Fig. 4). No difference in the number of neoformed vessels was found among the groups. Although **Group B** presented a significant variation in the amount of collagen (KW $p = 0.014$), of fibroblasts (KW $p = 0.038$) and of myofibroblasts (KW $p = 0.027$) over the time, no difference was found among the groups in every time studied. **Group B** showed a significant increase in the formation of neurofilaments over the time (KW $p = 0.03$); however, no difference was found among the groups in the times studied (Fig. 5). When correlating presence of the neurofilament protein and presence or absence of muscle layer criteria, the repaired muscle layer in **Group B** was noted to resemble the architecture of a normal bladder, with a more adequate neural network on postoperative day 28 (Fig. 6).

None of the animals killed on postoperative day 28 showed SRY mRNA expression (Fig. 7). The histological and immunohistochemical findings were summarized in Table 2.

Discussion

Tissue engineering technology makes it possible to rebuild a functional urinary bladder. In cases in which muscle cells are not available, or are phenotypically inadequate, stem cells come up as an excellent alternative. MSCs are multipotent cells derived from a number of sources, among which is the bone marrow. They can be terminally differentiated into cells, including osteoblasts, adipocytes and

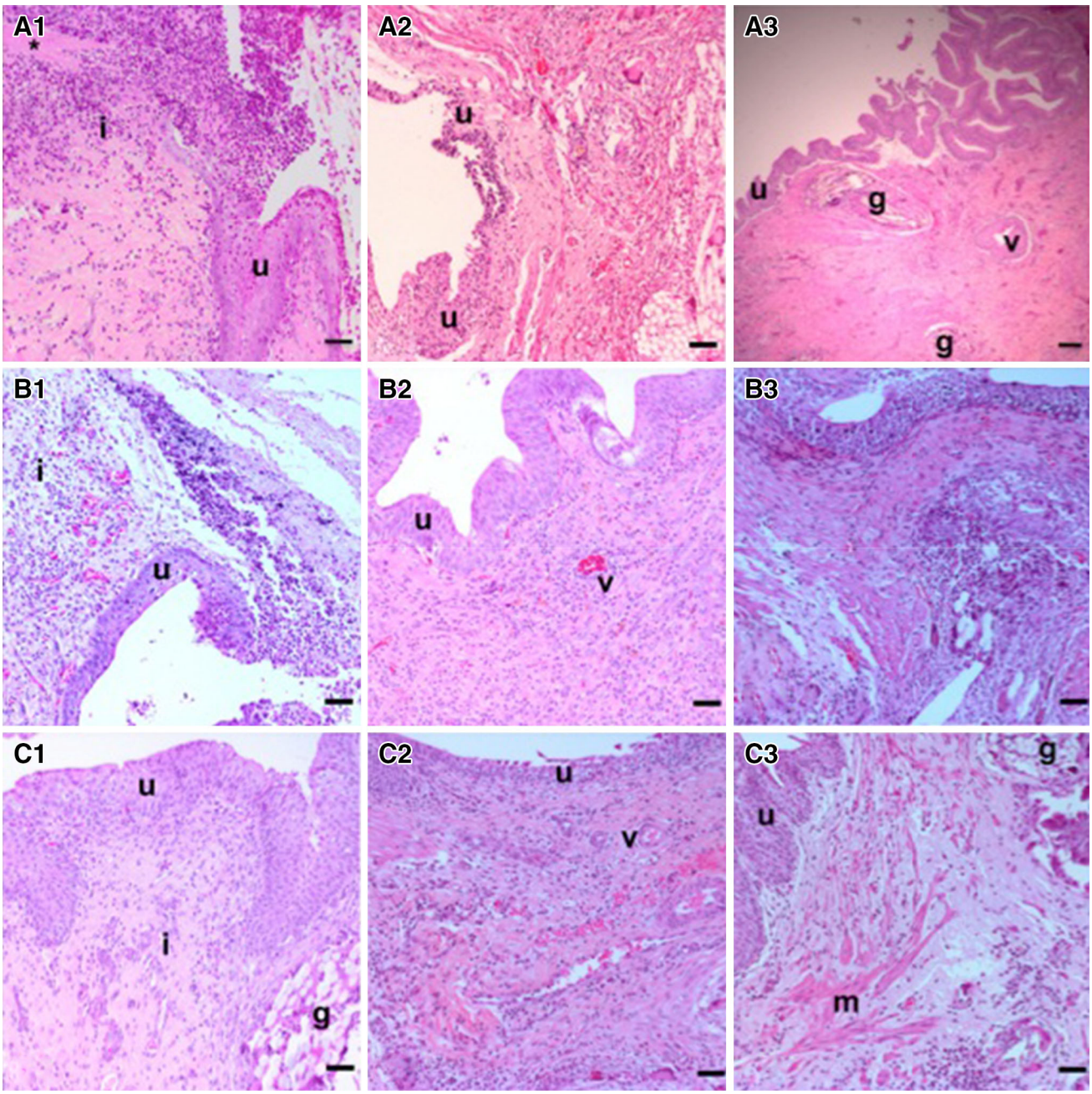

Fig. 1 Photomicrography of neobladders of the animals in **Groups A**, **B** and **C** killed on days 7 **(1)**, 14 **(2)**, and 28 **(3)** (H&E) (×100 and ×25 in **A3**). **A1–C1** Disorganized stroma with edema and dense inflammatory infiltrate; early stage of epithelial repair. **A2–C2** Epithelium already in its integrity and stroma with fusiform cells; moderate amount of capillary vessels. **A3–C3** Stroma with collagenous fibers, few cells and small number of capillary vessels. *u* urothelium, *g* foreign body granuloma, *v* vessels, *i* inflammatory cells, *m* muscle cells. *Asterisk* indicates decellularized bladder matrix

chondrocytes. MSC plasticity can differentiate into bladder cells as well as into cells derived from other embryonic layers, including neurons. MSCs have no MHC class II cell surface markers, but rather only MHC class I and no co-stimulator molecules, and are, therefore, invisible to the immune system of the host [16]. The MSC immunomodulator potential has been well studied. They can inhibit the action of T, B and natural killer cells and of antigen-presenting cells, through a paracrine secretion mechanism [17]. Such effects can be noted in experimental studies in which neural, pulmonary and renal injuries were improved using intravenous administration of MSCs [18–21]. Almost 70 % of the injected MSCs are retained in the lungs [22, 23]. Once in the blood stream, the MSCs are attracted to

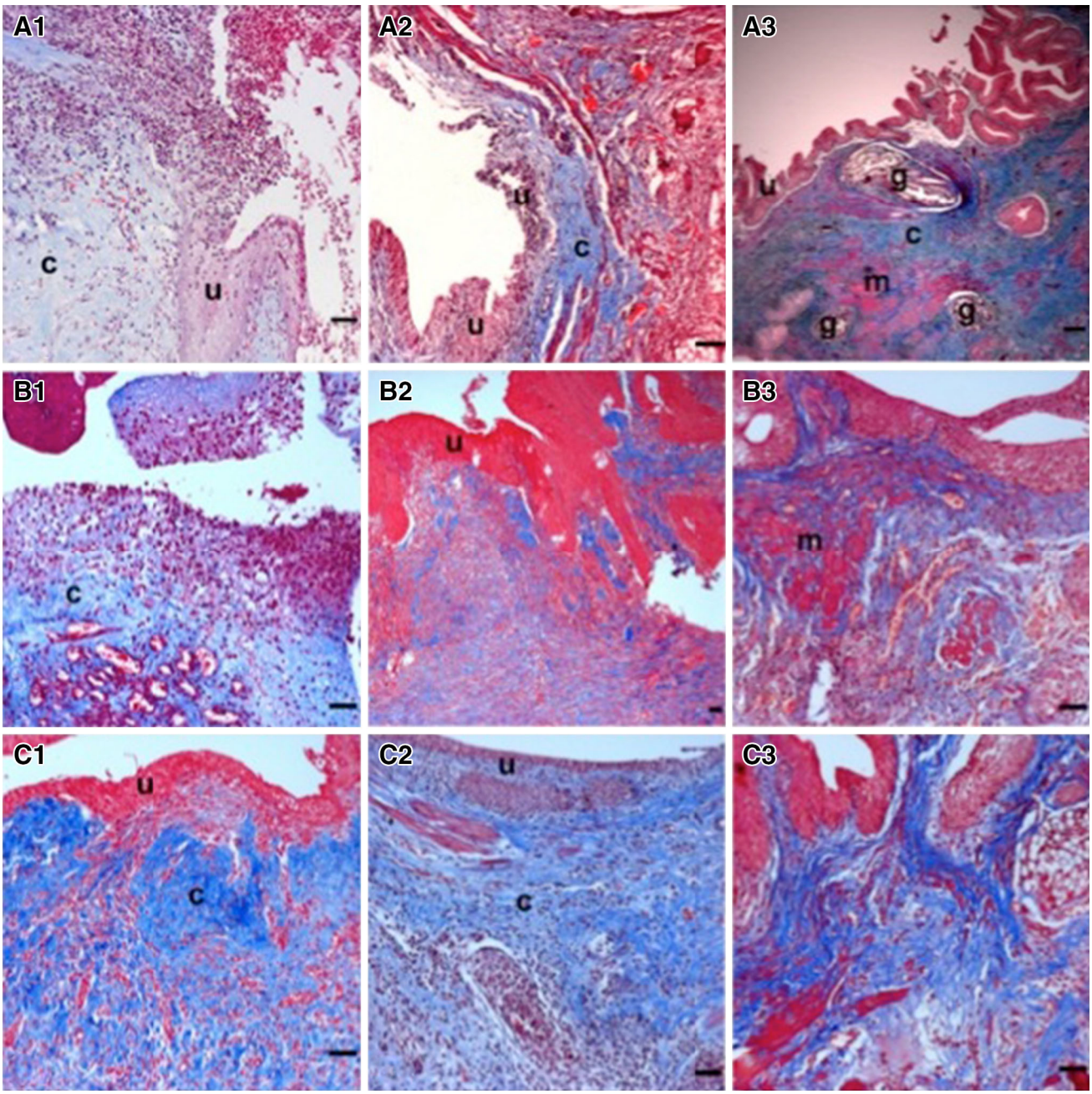

Fig. 2 Photomicrography of neobladders of animals in **Groups A**, **B** and **C** killed on days 7 **(1)**, 14 **(2)**, and 28 **(3)** (Masson trichrome) (×100; ×50 in **A3** and **B2**). **A1**–**C1** Disorganized and swollen stroma with neoformed capillary vessels and exuberant endothelium. **A2**–**C2** Stroma with fusiform cells arranged in bundles running parallel to the epithelium, intermingled with a moderate number of capillary vessels. **A3**–**C3** Stroma with collagen fibers and a small number of capillary vessels with plain endothelium lining. *c* collagen, *v* vessels, *u* urothelium, *g* foreign body granuloma, *v* vessels, *m* muscle cells. *Asterisk* indicates bladder decellularized matrix

the injured organs that must express specific chemoattractant receptors that facilitate this migration, adhesion and cell infiltration [23–26]. Nevertheless, systemic administration of MSC in bladder tissue engineering has not been tested yet. In this experiment the urothelium integrity was fully restored on postoperative day 14 in all groups studied. It can be said that the presence of MSCs, both when seeded in the matrix and when injected intravenously, had no influence on the urothelium regeneration, which was similar in all groups. These findings were observed in other experimental models that used stem cells seeded in extracellular matrices [10–13]. The muscle layer of the bladder is responsible for the property of storing large volumes without altering the intravesical pressure. Regeneration of

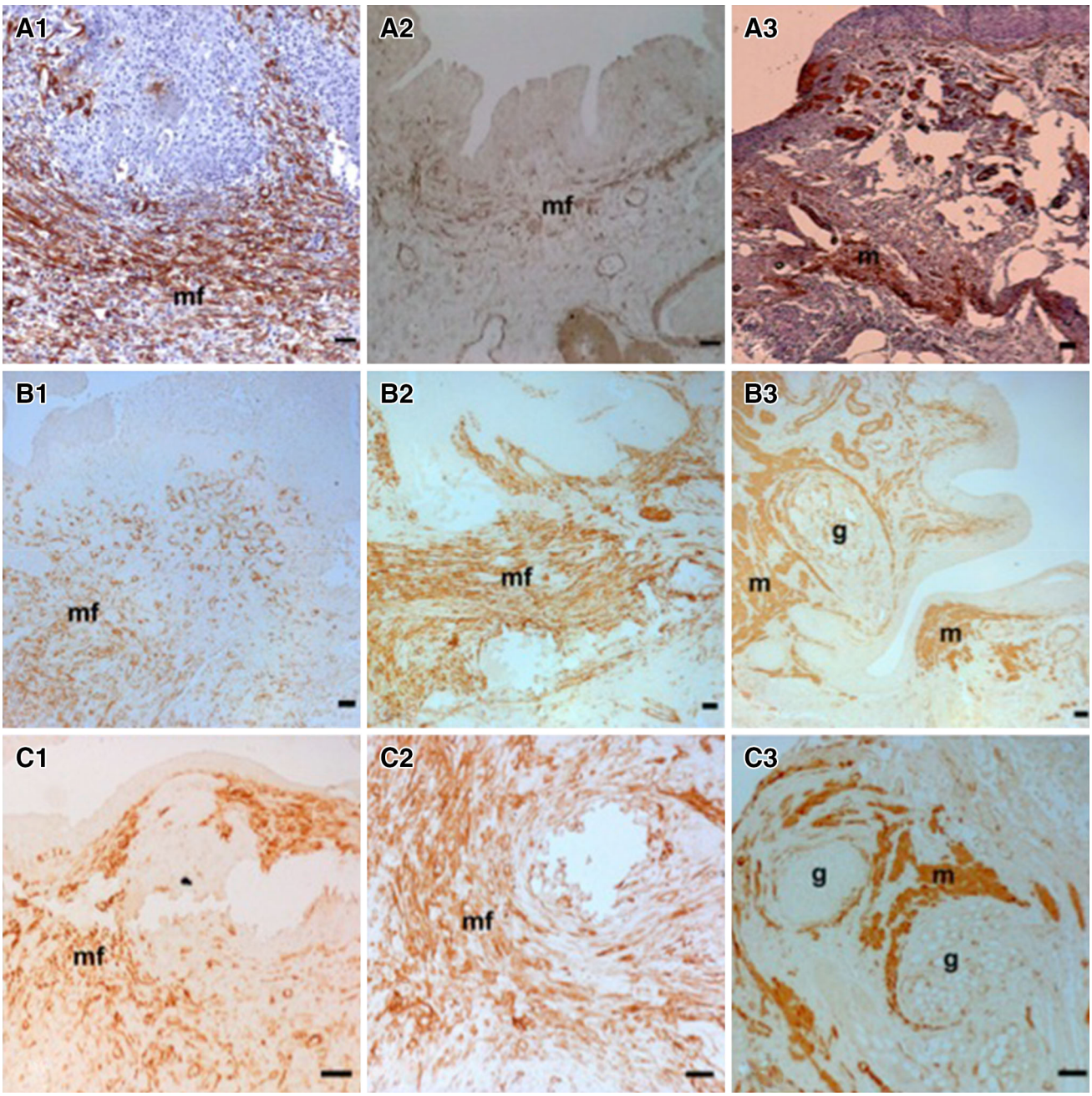

Fig. 3 Photomicrography of neobladders of animals in **Groups A**, **B** and **C** killed on day 7 **(1)**, 14 **(2)**, and 28 **(3)** (anti alpha actin) (×100, ×50 in **B1** and **B2** and ×25 in **A3**). **A1–C1** Myofibroblasts in bundles running parallel to the stroma. **A2–C2** Fusiform cells in the stroma. **A3** Disorganized muscle cells arranged into bundles. **B3**, **C3** muscle cells distributed in better organized bundles. *g* foreign body granuloma, *v* vessels, *mf* myofibroblasts, *m* muscle cells

the bladder smooth muscle is still little understood. Baskin et al. [27] showed that the interaction between the epithelium and the mesenchymal tissue is necessary for the development of the smooth muscle. Zhang et al. [4] also observed that presence of the urothelium makes a significant impact on the proliferation of the smooth muscle cells, since active penetrance of the matrix does not occur unless urothelial cells are grown in conjunction with smooth muscle cells. On the other hand, Kanematsu et al. [28] argued that the growth factors found in the urothelial cells culture media to be predominantly angiogenic and that the urothelium is, actually, little involved in the maturation of the muscle tissue. Their main role is believed to be the promotion of an adequate neovascularization, thus creating a more auspicious environment for the regeneration of muscle and urothelial cells in the graft. Although a

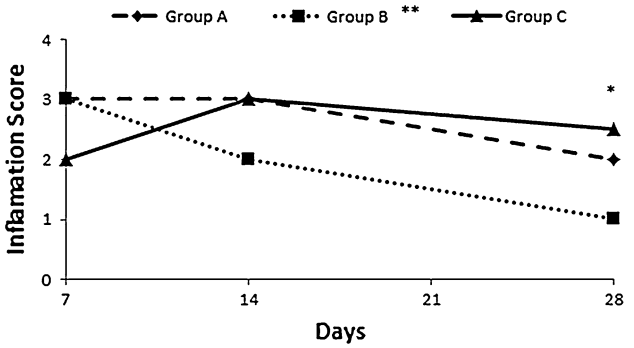

Fig. 4 Evolution of the inflammatory process in **Groups A**, **B** and **C** (median values). *Asterisk* indicates Kruskal–Wallis between groups on day 28, $p = 0.011$. *Double asterisk* indicates Kruskal–Wallis on the evolution of group B, $p = 0.003$

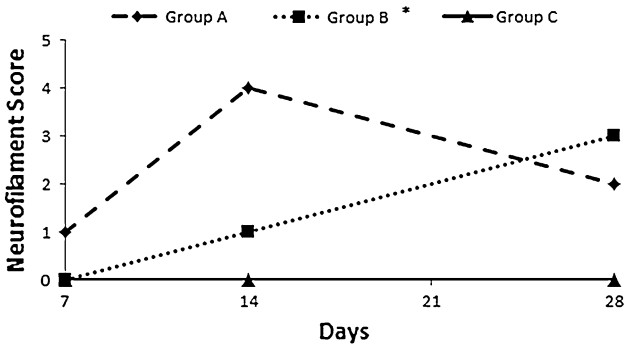

Fig. 5 Analysis of the formation of neurofilaments over the time in **Groups A**, **B** and **C** (median values). *Asterisk* indicates Kruskal–Wallis on the evolution of group B, $p = 0.03$

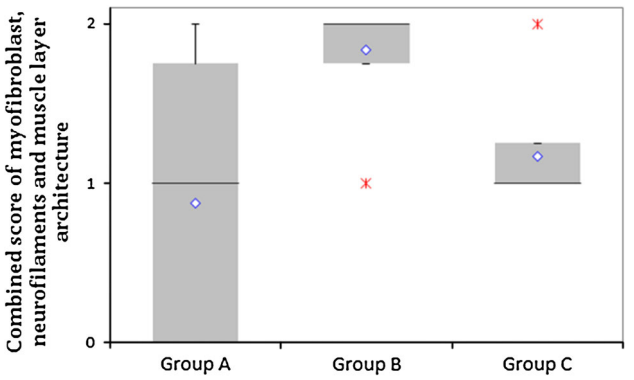

Fig. 6 *Boxplot* analysis of the combined score of myofibroblast, neurofilaments formation and muscle layer architecture variables on postoperative day 28 in the animals on **Groups A**, **B** and **C**. *Asterisks* are outliers and *diamonds* are mean values (GB > GA Mann–Whitney $p = 0.031$, GB > GC Mann–Whitney $p = 0.038$)

phenotypic change can be promoted in MSCs using muscle cells conditioned medium in experiments performed in vitro, the investigations that used extracellular matrices seeded with MSCs in animals were unable to prove that the muscle cells in the operated area were originated exclusively from their differentiation. Chung et al. [10], while studying acellular matrices seeded with bone marrow stem cells used in rats undergoing bladder augmentation, found that the muscle layer was already organized between weeks 2 and 4, while in the animals whose matrix had not been seeded, the muscle layer would not appear until the end of the third month. Zhu et al. [13] on the other hand, observed, in experiments on rabbits, no formation of a muscle layer similar to that of the native bladder until after 24 weeks, when the bladder augmentations were performed with acellular matrices seeded with adipose-derived stem cells. The animals in the control group, i.e., in which the matrices received no stem cells, showed organization of the smooth muscle cells in the grafts periphery. Zhang et al. [11] performed bladder augmentations with SIS seeded with bone marrow stromal cells in dogs. They observed muscle layer regeneration in the 10th week, with a result similar to that of the animals that had undergone seeding of urothelial and muscle cells. A common finding in those experiments was an earlier regeneration of the muscle layer regardless of the animal used. In this experiment, the muscle layer architecture was found to resemble that of the native bladder after 28 days in the groups using MSCs. Therefore, it was not possible to demonstrate the SRY mRNA expression in the neobladder, i.e., the MSCs were actually attracted to the graft areas, secreted their trophic factors and then suffered apoptosis (touch and go effect) [9, 29]. The trophic and anti-inflammatory factors secreted by the MSCs may lead to a more favorable environment for the regeneration and repair of the bladder wall. The partially organized muscle layer in **Group B** (MSC injection) suggests the immunomodulatory and anti-inflammatory properties of the MSCs as being more important in bladder repair than actually its cell fusion and differentiation capacity. No difference was found in the capacity for new vessels formation in the different groups. The best explanation for this fact may be that the early urothelium restoration process may have led to the secretion of angiogenic factors such as the bFGF (fibroblast growth factor-beta) and VEGF (vascular endothelial growth factor) which may have contributed for similar neovascularization in the groups [28]. **Group B** showed a progressive increase of fibroblasts and collagen in the first weeks, which may probably be explained by the beneficial and immunomodulatory effect of the exogenous MSCs. Because in this experiment we sought to observe only the effect of MSCs at the early stages of restitution it was not possible to demonstrate their possible benefits in fibrogenesis. Besides the muscle and urothelial architecture restoration, the neuronal network integrity must be observed if the new organ is to possibly exercise its function properly. In this experiment, a progressive increase in neural filaments over

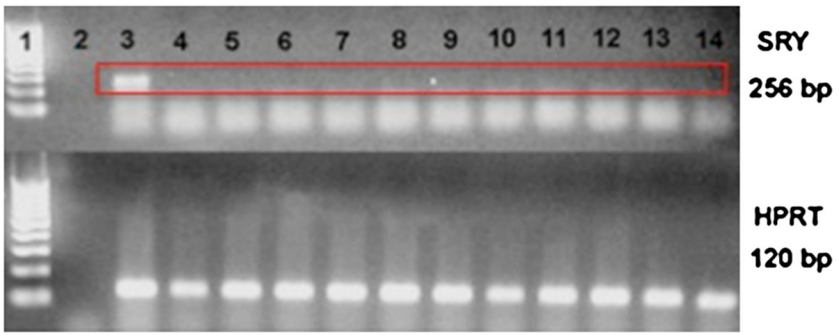

Fig. 7 SRY mRNA expression on postoperative day 28 in female rats treated with mesenchymal stem cells obtained from male rats, using the semi-quantitative PCR. *1* molecular weight, *2* Blank control, *3* Male rat bladder. *5*, *8*, *10* Bladders of animals from **Group A** (BAM augmentation). *4*, *6*, *14* Bladders of animals from **Group B** (BAM augmentation and MSCs IV administered). *7*, *9*, *11* Bladders animals from **Group C** (BAM-MSC seeded augmentation). HPRT mRNA expression as the "housekeeping gene". None of the animals killed on postoperative day 28 showed SRY mRNA expression

Table 2 Summary of histological and immunohistochemical findings in animals in **Groups A**, **B** and **C**

	Group A (decellularized heterologous matrix)	Group B (IV injection of mesenchymal stem cells)	Group C (decellularized heterologous matrix seeded with mesenchymal stem cells)
Inflammation	No inflammation reduction over the time PO 28: inflammation similar to Group C	Inflammation reduction over the time PO 28: presented the least inflammation among the groups	No inflammation reduction over the time PO 28: inflammation similar to Group A
Muscular layer	Disorganized architecture	Architecture similar to that of the native bladder	Architecture similar to that of the native bladder
Innervation	Absence of well formed neural network	Presence of well formed neural network	Absence of well formed neural network

the time was found only in **Group B** (MSC injection). As those animals received three MSC intravenous injections, their cumulative effect may have been important to lessen the inflammatory reaction and promote proper neural development. The inflammatory process was similar in the animals in Groups A and C, which may be connected with the host's immunological response to the MSCs differentiated in the matrices, before being incorporated into the bladder, considering that in the design studied, outbred rats were used. In the near future, we plan to conduct the same studies using animals of isogenic strain and medium/large animals, in which autologous MSCs can possibly be cultivated, as well as to assess in the long term the functional behavior of those bladder segments.

More than a differentiation or transdifferentiation, the exogenous MSCs, through their anti-inflammatory and immunomodulator properties, seem to have made the environment more favorable for regeneration of the neobladder walls and even contributed for the formation of a more adequate neural network. Longer term studies will certainly be necessary if we are to understand the biological mechanisms that lie behind these findings and to confirm the possible functional benefits from the use of MSCs in tissue engineering.

Conclusion

The use of MSCs contributes significantly for bladder regeneration and repair. Although their action has not interfered in the urothelium repair process, they were able to improve repair of the muscle layer. When intravenously injected, they triggered the neuronal regeneration process and, therefore, were more effective as compared when directly seeded in the matrices.

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Conflict of interest None.

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