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Mesenchymal stem cell therapy in a rat model of birth-trauma injury: functional improvements and biodistribution

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Abstract

Introduction and hypothesis—We evaluated the potential role of human mesenchymal stem cells (hMSCs) in improvement of urinary continence following birth-trauma injury.

Methods—Human MSCs were injected periurethrally or systemically into rats immediately after vaginal distention (VD) ($n=90$). Control groups were non-VD (uninjured/untreated, $n=15$), local or systemic saline (injection/control, $n=90$), and dermofibroblast (cell therapy/ control, $n=90$). Leak-point pressure (LPP) was measured 4, 10, and 14 days later. Urethras were morphometrically evaluated. In another sets of VD and non-VD rats, the fate of periurethrally injected hMSC, biodistribution, and in vivo viability was studied using human Alu genomic repeat staining, PKH26 labeling, and luciferase-expression labeling, respectively.

Results—Saline- and dermofibroblast-treated control rats demonstrated lower LPP than non-VD controls at days 4 and 14 ($P<0.01$). LPP after systemic hMSC and periurethral hMSC treatment were comparable with non-VD controls at 4, 10, and 14 days ($P>0.05$). Local saline controls demonstrated extensive urethral tissue bleeding. The connective tissue area/urethral section area proportion and vascular density were higher in the local hMSC- versus the saline-treated group at 4 and 14 days, respectively. No positive Alu-stained nuclei were observed in urethras at 4, 10, and

14 days. PKH26-labelled cells were found in all urethras at 2 and 24 h. Bioluminescence study showed increased luciferase expression from day 0 to 1 following hMSC injection.

Conclusions—Human MSCs restored the continence mechanism with an immediate and sustained effect in the VD model, while saline and dermofibroblast therapy did not. Human MSCs remained at the site of periurethral injection for <7 days. We hypothesize that periurethral hMSC treatment improves vascular, connective tissue, and hemorrhage status of urethral tissues after acute VD injury.

Keywords

Mesenchymal stem cell; Stress urinary incontinence; Stem cell fate; Birth-trauma injury; MSC biodistribution

Introduction

Urinary incontinence (UI) is one of the most prevalent conditions, affecting approximately 40% of women in the United States and the Western world. Stress urinary incontinence (SUI) accounts for the largest portion of these women [1]. It is likely that a combinations of anatomical, physiological, genetic, lifestyle, and reproductive factors interact throughout a woman's life span to contribute to pelvic floor disorders, including SUI [2]. Vaginal delivery can injure nerve, muscle, and connective tissues responsible for maintaining continence [3]. It appears that the imbalance in the degree of injury and potential recovery that occurs during and after vaginal birth is intimately related to the pathophysiology of SUI [2]. Despite the common use of midurethral slings for the treatment of SUI, this procedure is associated with a high level of complications and there is therefore a need for physiologic treatment options. Cell therapy and tissue engineering are among the newest strategies under investigation for the treatment and prevention of SUI [4–6]. The therapeutic use of stem cell therapy in SUI is based on two broad approaches: (1) skeletal muscle regeneration using adult muscle-derived progenitor-cell therapy after injury, and (2) repair/prevention using stem cell therapy at the time of injury or initiation of the phenotype [7–9]. While the first approach relies on the ability of the stem cell to engraft and differentiate, the reparative approach depends on the paracrine and anti-inflammatory function of the stem cell. Demonstration of the fate of stem cells once injected is therefore intimately tied to the establishment of efficacy based on the reparative approach.

We hypothesized that human bone-marrow-derived mesenchymal stem cell (hMSC) therapy immediately after injury would improve continence function. The vaginal distension (VD) model in female rats simulating birth-trauma-related injury to pelvic organs and resulting in dysfunction of the continence mechanism is one model that has been used as a preclinical model to explore the potential for cell therapies for SUI [7, 9, 10]. The pattern of injury and recovery described in these studies parallels the observed epidemiological pattern of SUI occurrence following vaginal delivery described by Viktrup et al. [11]. In this study, we investigated the efficacy, role, and fate of both periurethral and intravenous administered hMSC therapy in VD rats by functional measurements [leak-point pressure (LPP)], morphometric experiments, and bioluminescence imaging (BLI).

Materials and methods

Animal model, treatments, and measurements

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University (IACUC s: 2012–0139 and 2010–0120). Of 285 female Sprague–Dawley rats, 270 underwent serial VD under 90 mg/kg IP ketamine–xylazine-induced anesthesia with lubricated 24- to 32-F bougie dilators, followed by a modified 10-F Foley catheter gradually inflated with 3 cc sterile water and secured in place for 4 h [11]. At the end of VD, rats received treatment with either periurethral ($n=135$) or systemic ($n=135$) injection of $0.1\text{ ml}:10^6$ hMSCs ($n=45$) or 10^6 rat dermofibroblasts ($n=45$) or normal saline ($n=45$). Fifteen rats were preserved as controls (uninjured/untreated). At 4, 10, or 14 days ($n=15$ for each group in each time point) after VD, a mean of 8–12 LPP measurements were taken via an implanted suprapubic tube [10].

Dosage of cell therapy (10^6 cell/rat) was selected based on previous experiments in rat incontinence models, which demonstrated increase in LPP with increases in injected cell number and evidence of obstruction in the high-dose (10^7 cells) groups [12]. Human MSCs were not expected to be rejected by receiver rats because of their known immunomodulatory effect [13]. In addition, rat-derived dermofibroblasts were included as an additional control to evaluate if possible therapeutic effect is MSC-specific or any other cell types can have therapeutic effects on SUI.

Source and preparation of hMSC, and dermofibroblast

Human MSCs were isolated from bone marrow aspirated from the iliac crest of healthy human donors (age 17–58 years) after informed consent under a protocol approved by the Institutional Review Board at University Hospitals of Cleveland (IRB 09-90-195, approved 3 December 2012), according to previously published methods [14]. Rat dermofibroblasts were derived from the papillary dermis of a Sprague–Dawley rat, as described by Schafer et al. [15].

Histological analysis

Immediately after LPP measurement, the entire urethra of six anesthetized rats from each group was dissected from surrounding tissue and fixed with 4% paraformaldehyde for histology evaluation. Serial sections (3 mm) were obtained from proximal, distal, and midurethra for hematoxylin and eosin (H&E), trichrome, or immunohistochemistry (IHC). The “descriptive” histology of the urethra included comparison between groups of edema, inflammation, and bleeding on H&E-stained slides and connective tissue and vasculature on trichrome-stained slides. The extent of connective tissue correlated inversely with the remaining muscle on each slide.

Quantitative histology of the urethra was performed using Image-Pro Plus software (Version 7.0, Media Cybernetics, Silver Spring, MD, USA). For each animal, two midurethral sections were randomly chosen for analysis. In each cross-section, trichrome-stained images were quantified for the thickness of urothelial layer, smooth muscle, skeletal muscle, and connective tissue area in pixels. In each urethra, we calculated:

1. Average proportions of urothelial-layer area to whole urethral-tissue area
2. Smooth muscle to whole urethral-tissue area
3. Skeletal muscle to whole urethral-tissue area
4. Connective tissue within smooth-muscle area to the smooth-muscle area
5. Connective tissue within skeletal-muscle area to the skeletal-muscle area

For analysis of vascular density, the ratio of the circumference of total vascular structures to total urethral tissue cross-section area was determined for randomly selected urethras from control or treatment groups 14 days after VD.

Immunofluorescent and histochemical staining of urethral tissue

As functional studies demonstrated the best SUI improvement results in locally treated groups, we performed complimentary histopathologic studies on their midurethral tissue samples. To confirm results of muscle-layer-thickness quantification, three midurethral samples were randomly selected from urethras harvested 14 days after local treatments. They were then treated with 1:500 dilution of mouse anti-fast myosin skeletal heavy chain (Abcam, Cambridge, MA, USA) followed by 1:2000 dilution of biotinylated goat anti-mouse immunoglobulin-G (IgG) (Santa Cruz Biotechnology, Dallas, TX, USA). Slides were developed conventionally using the avidin–biotin complex-detection (ABC) method (Vector Laboratories, Burlingame, CA, USA), counterstained with hematoxylin-QS (Vector), and imaged under light microscope.

To confirm results of vascular-density quantification, three samples were randomly selected from the urethras harvested 14 days after local treatment and treated with 1:100 dilution of rabbit anti-CD31+ antibody against vascular endothelial cells (Santa Cruz Biotechnology) followed by 1:2000 Alexa 568 goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA), mounted with 4',6-diamidino-2-phenylindole (DAPI) and examined under fluorescence microscope.

Statistical analysis

Continuous variables are reported as means±standard deviation (SD), and the level of significance between groups was determined using analysis of variance (ANOVA) with post hoc Tukey test ($P<0.05$). A test of homogeneity of variances was performed. Blinded LPP measurement and histological and morphometric analysis was performed based on animal numbers.

Fate of injected hMSCs

In situ histopathological tracking of hMSCs 4, 10, and 14 days after VD—To detect transplanted hMSCs in MSC-treated groups, nuclei were traced using in situ hybridization (ISH) for human Alu genomic repeats via digoxigenin (DIG)-labeled DNA probes. Midurethral and distal-urethral tissue sections of five randomly selected animals from each time point of locally or systemically hMSC-treated groups were labeled for human-specific Alu [16]. Also, one rat was sacrificed 2 h after receiving 10^6 hMSCs

periurethrally, and we performed ISH on the urethral tissue as a positive control for Alu-stained periurethral hMSCs.

In situ tracking of fluorescent-labeled hMSCs 1 day after VD—Eight female Sprague–Dawley rats underwent VD and received 10^6 PKH26-labeled hMSCs periurethrally ($1 \mu\text{mol/L}$ fluorescent chromophore, Sigma). Rats were sacrificed 2 h ($n=4$) and 24 h ($n=4$) after receiving labeled hMSCs. Proximal, middle, and distal sections of urethras were studied under immunofluorescent microscope. To confirm the results, the positive slides for PKH26 underwent ISH for human Alu genomic repeats [16].

In vivo assessment of hMSCs by BLI—To track hMSC after local periurethral injection in vivo in real time, and to prove cell viability, we transfected hMSCs with a triple-fusion imaging reporter system [17] selected primarily for its luciferase expression, which enables in vivo BLI to assess hMSC viability and distribution. Seven rats underwent VD and received 1–1.5 million luciferase-expressing hMSCs periurethrally through a vaginal approach immediately after VD. Three control rats also received local periurethral injection of 1.5 million luciferase-expressing hMSCs but did not undergo VD.

For BLI, rats were first anesthetized with isoflurane gas 2 h after hMSCs injection and then given a single injection containing 0.8 ml of 50 mg/ml d-luciferin potassium salt dissolved in phosphate-buffered saline (PBS). Ten min after luciferin injection, the rats were imaged on a Xenogen IVIS 200 bioluminescence scanner (PerkinElmer). Images were collected for 30 s, 1 min, 3 min, and 10 min to improve detectability of stem cells. BLI data was obtained immediately following hMSC inoculation and at days 1 and 2 following inoculation. Animals were sacrificed 7 days after hMSC injection, and the urethra was dissected out and underwent human Alu genomic repeat staining to confirm BLI results histopathologically.

Results

Efficacy of hMSC therapy of VD incontinence model

A mean of 12 of 15 animals at each time point and in each treatment group underwent LPP measurement. The remaining animals had either incompetent suprapubic tube or they did not survive after VD or suprapubic-tube-placement procedures. After VD, mean LPP in rats treated systemically with saline (18.82 and 20.77 mmHg; $P=0.01$), but not hMSCs (26.57 and 27.84 mmHg; $P=0.53$ and $P=0.21$, respectively), was significantly lower than in control rats (28.56 mmHg) at 4 and 10 days, respectively. By day 14, LPP in rats treated systemically with saline after VD showed recovery of continence, as expected in a VD model ($P=0.14$), and LPP in rats treated systemically with hMSCs remained comparable with non-VD controls ($P=0.36$; Fig. 1). Rats treated with periurethral injection of hMSCs after VD demonstrated mean LPP similar to control rats at 4, 10, and 14 days ($P=0.46$, 0.83, 0.92, respectively). Periurethral injection with saline at 4 and 14 days yielded mean LPP significantly lower than that in control rats ($P<0.01$ at 4 and 14 days). In this group, there was no statistically significant difference in mean LPP vs. controls at 10 days ($P=0.17$). VD animal groups treated with rat dermofibroblast systemically and periurethrally demonstrated a mean LPP significantly lower than the untreated control rats ($P<0.01$) at all time points (Fig. 1).

Histological analysis

Gross morphological observations of H&E- and trichrome-stained urethral sections in animals treated with local saline injection showed extensive bleeding in muscular layers and connective tissue around the lumen of the urethra 4 days after and to a lesser extent 14 days after VD. In dermofibroblast- and hMSC-treated groups, the same pattern of bleeding throughout the urethral wall layers was visible, although with much smaller pools of blood 4 days after VD; almost no bleeding could be found 14 days after VD (Fig. 2a and b). Edema and inflammation were not seen among any group at different times postinjection. Saline- and dermofibroblast-treated groups had minimal connective tissue at day 4 but increased connective tissue at day 14 compared with previous time points and controls. Human MSC-treated groups showed more pronounced connective tissue compared with the saline-injected group at days 4 and 10 but not at day 14.

Quantitative analysis of defined morphometric factors in midurethral sections showed a significantly higher ratio of total connective tissue area to total urethral tissue area in hMSC-treated animals treated locally compared with saline-treated animals 4 day after injury [$P=0.026$, 95% confidence interval (CI)=0.017, 0.351, respectively]. Quantitative analysis of microvascular structures by Image-Pro software 14 days after VD demonstrated an increase in vasculature in the urethra of VD rats treated locally and systemically with hMSC compared with saline-treated VD animals and control animals ($P<0.05$ for all comparisons). Changes in microvascular structure after dermofibroblast treatment locally and systemically were not significant (Fig. 3a).

Immunofluorescent and histochemical staining of urethral tissue

Muscle thickness, as measured by the proportions of myosin-positive areas (skeletal muscle) in midurethras, was comparable in controls, saline-, hMSC-, and dermofibroblast-treated groups 14 days after treatments (Fig. 2b). Systemic and local hMSC treatment and local dermofibroblast treatment demonstrated markedly higher CD31-positive vascular structures in the urethra compared with the saline group 14 days after treatment (Fig. 3b).

Fate of injected hMSCs

In situ histopathological tracking of hMSCs 4, 10, and 14 days after VD—No positively Alu-stained nuclei were observed in serial transverse sections of distal, proximal, and midurethral urethras harvested 4, 10, and 14 days after VD in locally and systemically hMSC-transplanted groups (Fig. 4a-1). The positive control urethra, collected 2 h after hMSC injection, clearly revealed Alu-positive hMSCs around the urethra, while cells of the underlying recipient rat tissue did not stain (Fig. 4a-2).

In situ tracking of fluorescent-labeled hMSCs 1 day after VD—PKH26-labelled cells were found in all grafted sphincters at 2 and 24 h after injection, mostly in midurethral sections (Fig. 4b-1 and b-2). A population of coalesced cells was retained in connective tissue between urethra and vagina 24 h after VD.

In vivo real-time tracking of cells by BLI imaging—Longitudinal BLI from rats that underwent VD and received a local periurethral injection of luciferase-expressing hMSCs

showed a visible increase in BLI signal on days 1 and 2 in comparison with day 0 (Fig. 5a), immediately following hMSC injection. VD rats showed a significant increase ($P<0.05$) in BLI signal intensity at days 1 and 2 postinoculation. No temporal difference in BLI signal was observed for the non-VD rats (Fig. 5b). BLI signals were attenuated by day 7 (data not shown).

No positively Alu-stained nuclei were observed in the serial distal, proximal, and midurethral transverse plane sections of harvested urethras at the end of imaging, confirming the disappearance of BLI signal; however, in the positive-control urethral sample collected 2 h after hMSC injection, staining for human-specific Alu repeats clearly revealed human cells by their nuclear staining (Fig. 5c).

Discussion

This study demonstrated a remarkable improvement in urethral continence function and amelioration of VD injury after local or systemic injection of hMSC. Improvement was specific to the injection of hMSC, since the injection of saline or dermofibroblasts did not have a similar effect on LPP measurements. Although this study of heterologous MSCs and numerous other studies of homologous MSCs indicate that MSCs produce favorable functional outcomes in animal models with SUI, the mechanism of action remains poorly understood [18]. In the study we report here, morphometric evaluation of urethra and fate of hMSCs provided insights into potential mechanisms of action of hMSCs in improving LPP in this VD model of birth-trauma injury. Previously, Kinebuch et al. [19] injected rat autologous MSCs, and Corcos et al. injected rat homologous MSC periurethrally into urethral/ periurethral tissue, respectively demonstrating the amount of skeletal or smooth- and skeletal-muscle increased in urethra. In contrast, our results did not demonstrate a difference in the quantity of the skeletal or entire muscle layer at the midurethral level after the injection of hMSCs. However, this inconsistency between our observation and the two studies mentioned may be the result of difference in animal models or MSCs (rat MSCs vs hMSCs).

The reduced bleeding observed after injury and hMSCs treatment in our study, as well as the increased connective-tissue-layer thickness and microvascular density in the urethra, all suggest a possible paracrine/protective effect of hMSCs, where the degree of injury is ameliorated or repair is accelerated through neovascularization. Both VD and local therapeutic injections could cause bleeding in the urethra and the connective tissue around the urethra. The extensive bleeding pools 4 days after VD in urethral tissue of rats treated locally with saline in contrast to no bleeding or less bleeding seen in hMSC- and dermofibroblast-treated animals may be interpreted as a hemostatic effect of hMSCs and, to some extent, dermofibroblasts. This difference in hemorrhage may also be a consequence of vasoconstrictive substances being released from hMSCs, although this was not measured in our study. However, why hMSC hemostatic effect leads to urinary continence improvement as soon as 4 days after injury remains unknown. It should be noted that bleeding is most probably the consequence of VD, not local injections, because the site of injection was in the vaginal wall alongside the urethra, not in the urethral wall alone. Furthermore, injections

were performed on one side of the vaginal wall, while bleeding was equally dispersed all around the urethral tissue.

The lower proportions of total connective tissue area to total urethral tissue area in saline-treated compared with hMSC-treated animals demonstrates the presence of relatively more connective tissue in hMSC-treated animals 4 days after treatment. This higher proportion of connective tissue in urethras from hMSC-treated rats a few days after VD is consistent with previous studies demonstrating increased urethral connective tissue content after umbilical cord hMSC treatment in longer period post-VD [20]. Increased connective tissue content may demonstrate a mechanism of tissue repair and may also provide an explanation for restoration of UI in animals treated locally with hMSC. However, systemically treated animals did not show this pattern of histological difference when compared with saline-treated animals. Additionally, the ratio of vascular density to whole urethra area was significantly greater in the hMSC group compared with the saline, dermofibroblast, and control groups. This higher level of vascularization is consistent with Du et al. [6] who reported results after implantation of MSC-composite gel; it also demonstrates another mechanism of tissue repair or improvement orchestrated by hMSCs or their secretory factors, which induce neovascularization or vascular preservation in the acutely injured tissue.

Although previous studies reported detection of few, if any, transplanted cells, one possible explanation is that histological assessment was often performed several weeks after initial cell transplantation; thus, most cells may have reached the end of their natural lifespan [21]. Our study showed no positive Alu-stained hMSC 4, 10, and 14 days after injection; additionally, in vivo BLI signal in VD animals increased within 1 day after injection and then started to decrease, disappearing in all animals by 7 days, validating the absence of cells 4 days after concomitant injury/stem-cell therapy in a VD model. Moreover, the presence of PKH-labeled cells 2 h and 24 h after injection and confirmation of their presence by human Alu staining is evidence for their proper periurethral injection. Furthermore, finding larger a proportion of coalescence of PKH26-positive cells 24 h compared with 2 h after VD, along with increased BLI signal 24 h post-VD, proposes the possibility of hMSC proliferation at the site of injection in the first 24 h, followed by systemic migration/or elimination before day 7. It is very important to explore the progression of events and their mechanism between the 1st and 4th days after injection, when cells may disappear from the site of injection. Studying the fate of injected cells at the site of injection illustrates that our results are compatible with those of Grinnemo et al. [22], who found hMSCs 2 days postinjection in the myocardium of Sprague–Dawley rats without immunosuppression, but not 7 days after injection. However, unlike the discovery of a massive macrophage infiltration at the site of hMSC transplantation in myocardium in the Grinnemo study, the rat urethral tissue did not show significant inflammatory-cell infiltration 1, 2, 4, 10, and 14 days after hMSC injection in our study. This could be because the transplanted cells were derived from different human donors with potentially different culture media components and were injected in different tissues. Also, previous exposure of rats to hMSC-related antigens would have been different in the two studies, which resulted in different types of immune reactions. Hence, when taken together, these findings and the lack of persistent hMSCs in the urethral area 4 days after systemic or local injection refute the hypothesis that MSCs injected into the

milieu of injury would incorporate, differentiate, and restore sphincter structure. Moreover, our findings regarding the fate of hMSCs and the histopathology of local hMSC-injected rat urethras contrast with the results of MSC long-term stability and differentiation at the site of injection, which has been reported in a number of previous studies [18, 19, 23_25]. Evidence that labeled cells disappear from the site of injury only a few days after injection, or the inability to detect cells pathophysiology model described suggests that it may be possible to “push” urinary incontinent patients back to the normal curve and prevent them from developing bothersome, long-lasting SUI, with improvements in pelvic floor strength/ reserve following the inciting injury of pregnancy and delivery, which can enormously improve their quality of life. Multipotent stem cells, by improving repair and prevention of destructive mechanisms at the time of injury or initiation of the phenotype, can be a therapeutic option for SUI therapy in women with VD. This reparative approach would depend on paracrine and anti-inflammatory functions of stem cells. Results of this study should be confirmed and completed by adding a sham VD group for all time points, standardizing the site of periurethral MSC injection, differentiating between postinjection and post-VD-induced urethral hemorrhage, and clarifying components of increased connective tissue post-MSC therapy by staining for different types of collagen, elastin, glycosaminoglycans, proteoglycan, hyaluronan, and fibroblasts, etc.

VD injury causes extensive bleeding in urethral tissue. Human MSCs injected systemically or periurethrally restored the continence mechanism with an immediate and sustained effect in this murine model for SUI. The transplanted hMSC improved vascularization and connective tissue status after acute VD injury. Periurethrally transplanted hMSCs stay at the site of injection for <7 days, and then disappear; hence, hMSC paracrine action can be a possible mechanism of SUI improvement rather than hMSC engraftment into tissues and in situ differentiation. Further investigation is required to explore mechanisms of injury and repair in VD and to explore hMSC treatment, moving toward clinical applications of hMSCs for treating SUI.

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Abbreviations

BLI	Bioluminescence imaging
hMSCs	Human mesenchymal stem cells
IHC	Immunohistochemistry
ISH	In situ hybridization
LPP	Leak-point pressure

SUI	Stress urinary incontinence
VD	Vaginal distention

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Fig. 1.

Efficacy of hMSC therapy in rat SUI model: **a** Rats treated with periurethral injection of hMSCs after VD demonstrated mean LPP comparable with control rats at 4, 10, and 14 days ($P=0.46, 0.83, 0.92$, respectively). Periurethral injection with saline at 4 and 14 days yielded mean LPP significantly lower than that in control rats ($P<0.01$ at 4 and 14 days). Dermal fibroblast injection did not improve LPP to a significant level. **b** Rats treated with systemic injection of hMSCs after VD demonstrated mean LPP comparable with control rats at 4, 10, and 14 days. Periurethral injection with saline at 4 and 14 days yielded mean LPP significantly lower than that in control rats ($P<0.01$ at 4 and 14 days). Dermal fibroblast injection did not improve LPP to a significant level. *rDF* rat dermal fibroblasts, *hMSC* human mesenchymal stem cells, *SUI* stress urinary incontinence, *VD* vaginal distension, *LPP* leak-point pressure.

Fig. 2.

Histological evaluation of locally treated rat urethral tissues: **a** Arrows in hematoxylin and eosin (H&E)-stained slides show extensive bleeding in muscular layers and connective tissue around the midurethral lumen 4 days after vaginal distention (VD) and **b** to a lesser extent 14 days after VD in saline-treated groups. **a** In dermofibroblast- and hMSC-treated groups a similar pattern of bleeding throughout urethral-wall layers is visible, although with much smaller pools of blood 4 days after VD; **b** almost no bleeding could be found 14 days after VD. **b** Comparing proportions of myosin-positive areas (skeletal muscle) in urethras,

muscles thickness is equal in control, saline-, hMSC-, and dermofibroblast-treated groups 14 days after VD. *hMSC* human mesenchymal stem cells, *VD* vaginal distension, *LPP* leak-point pressure, *SkM* skeletal muscle.

Fig. 3.

Urethral tissue vascular structure analysis: **a** Quantification analysis of microvascular structures by Image-Pro Plus software demonstrated increase in vasculature in the midurethra of VD rats treated with hMSC after 14 days in comparison with rats treated with saline and control rats ($p<0.05$). **b** Immunofluorescence urethral staining confirmed that CD31-positive blood vessels (*red structures, solid arrows*) in muscle or mucosa/submucosa were higher in animals treated with hMSC locally and systemically than those treated with dermofibroblasts and N/S. 4',6-diamidino-2-phenylindole (DAPI) shows nucleus in *blue*. VD vaginal distension, Sys systemic, Fib dermofibroblast, N/S normal saline

Fig. 4.

Fate of periurethrally injected hMSCs: **a** Digoxigenin (DIG)-labeled DNA probes corresponding to human-specific Alu repeats to detect hMSC in rat urethra: **a-1** No positively stained Alu nuclei were observed in serial transverse-plane sections of distal-, proximal-, and midurethral urethras 4, 10, and 14 days after VD in locally and systemically hMSC-transplanted groups. **a-2** Representative urethra 2 h after hMSC injection demonstrates hMSCs. DIG-labeled DNA probes corresponding to human-specific Alu repeats clearly reveal nuclear staining of human cells, whereas cells of the underlying recipient tissue (rat origin) did not stain; $\times 40$ light microscope imaging. **b** PKH26-labelled cells were found in all grafted sphincters at **b-1** 2 and **b-2** 24 h after injection mostly in midurethral sections. Urethras after 24 h showed a large population of cells seemingly proliferated and homed in connective tissue between urethra and vagina. *hMSC* human mesenchymal stem cells

Fig. 5.

BLI of hMSC localization in VD rats: **a** Representative longitudinal BLI images from a VD rat showing increasing BLI signal on days 1 and 2 following periurethral injection of luciferase-expressing hMSCs. **b** Mean BLI signal in VD animals significantly increased on days 1 and 2 ($P < 0.05$) in comparison with day 0, suggestive of hMSC recruitment/viability. No significant difference was observed for non-VD rats. **c** Representative urethra 2 h after hMSC injection demonstrates hMSCs: human-specific Alu repeats clearly revealed nuclear staining of hMSCs, whereas no positive Alu signal was found in urethra of imaged animals when BLI signal disappeared. Light microscopy $\times 40$. *BLI* bioluminescence imaging, *hMSC* human mesenchymal stem cells, *VD* vaginal distention