

## ORIGINAL ARTICLE

# Transplantation and repair: Combined cell implantation and chondroitinase delivery prevents deterioration of bladder function in rats with complete spinal cord injury

K Fouad<sup>1</sup>, DD Pearse<sup>2</sup>, W Tetzlaff<sup>3</sup> and R Vavrek<sup>1</sup>

<sup>1</sup>Faculty of Rehabilitation Medicine, University of Alberta, Edmonton, Alberta, Canada; <sup>2</sup>The Miami Project to Cure Paralysis, and Neurological Surgery, Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL, USA and <sup>3</sup>ICORD (International Collaboration on Repair Discoveries), University of British Columbia, Vancouver, British Columbia, Canada

**Study design:** Additional examination. In this study, we report changes in bladder function after a combined treatment that was designed to study axonal regeneration after complete spinal cord injury (SCI) in rats.

**Objectives:** To report effects on bladder function following the administration of a combined treatment for complete SCI.

**Setting:** University of Alberta, Faculty of Rehabilitation Medicine, Edmonton, Canada

**Methods:** Eight rats received Schwann cells in Matrigel-filled guidance channels, olfactory ensheathing glia and chondroitinase ABC at the lesion site following complete thoracic SCI. Controls ( $n = 7$ ) received Matrigel only. Daily bladder examinations were performed. Analysis of bladder size, wall thickness, actin and collagen type III was performed after 14 weeks.

**Results:** Following SCI, both groups regained bladder voiding after 3 weeks. However, 2 weeks later, incontinence was observed in all untreated rats and two treated rats. Post-mortem examination of bladders revealed enlarged bladder sizes. Thicker bladder walls were found in untreated rats, which were composed of disorganized bundles of smooth muscle fibers surrounded by high amounts of collagen (type III).

**Conclusion:** We show that the combined treatment prevents collagen deposition in bladder walls and maintains the rat's ability to void efficiently. Although the mechanism responsible for this improvement is unclear, our study shows that the present combinatory therapy can influence bladder function, thus expanding their utility as a broad reparative approach for SCI.

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**Keywords:** spinal cord injury; bladder; collagen; combined treatment; Schwann cells

## Introduction

In rats, spinal cord injury (SCI) rostral to L6 spinal segment diminishes voluntary bladder and external urethral sphincter control. Normal lower urinary tract function requires the central nervous system to coordinate the bladder and external urethral sphincter outflow. During micturition in rats, normal voiding depends on synchronous activation of bladder contraction and bursting activity of external urethral sphincter, thereby involving a complex interaction between the pontine micturition center, sympathetic and parasympathetic nervous system and motoneurons in the spinal cord.<sup>1,2</sup> SCI initially produces a period of bladder areflexia

with large residual urine volumes that cause bladder hypertrophy and overdistension, but eventually this period is followed by an emergence of a spinal micturition reflex that leads to continuous involuntary bladder contraction.<sup>3</sup> However, the continuous co-contractions of the bladder and the sphincter (bladder–sphincter dyssynergia) lead to inefficient voiding and large residual urine volumes.<sup>4</sup> Other factors contributing to inefficient voiding of the bladder include alterations in bladder size and tissue composition, including the accumulation of collagen type III.<sup>5,6</sup> As a result of continuous vesicular pressure and urine retention in spinalized rats, the phase of restored, but inefficient, bladder function might lead to complete deterioration of bladder compliance/function, infections and other lower urinary tract complications.<sup>7</sup>

We reported earlier that following complete thoracic (Th8) spinal cord transection in rats, a combination of bridging the

Correspondence: Dr K Fouad, Faculty of Rehabilitation Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2G4.

E-mail: karim.fouad@ualberta.ca

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lesion with Schwann cell-filled guidance channels, olfactory ensheathing glia implantation and chondroitinase ABC delivery promoted the regeneration of serotonergic fibers into the lumbar spinal cord and improved locomotor recovery.<sup>8</sup> We also showed that propriospinal interneurons and fibers from various brain stem nuclei, including vestibular, reticular and raphe nuclei, regenerated through the tissue bridge into the caudal spinal cord.<sup>9</sup> During these studies, originally designed to investigate motor recovery and axonal regeneration, we realized that the combined treatment also affected long-term bladder function. Thus, following completion of planned experiments, bladders from the same rats were examined, and notes taken during postoperative care were compared between the groups. As it was not our original intention to study bladder function, we recognize that all desired outcome measures could not be performed. However, we feel that the resulting findings are significant and could be of interest to the field.

## Materials and methods

Spinal cord lesions and combinatory treatment were performed according to Fouad *et al.*<sup>8</sup> using 19 adult female Fischer rats (Charles River, Wilmington, MA, USA). All procedures were approved by local authorities according to the guidelines of the Canadian Council for Animal Care.

Schwann cells and olfactory ensheathing glia were prepared as described earlier.<sup>10</sup> Before surgery, animals were anesthetized with Hypnorm (fentanyl citrate, 120  $\mu$ l per 200 g body weight; Janssen Pharmaceuticals, Beerse, Belgium) and Midazolam (0.75 mg in 150  $\mu$ l per 200 g body weight; S.C.; Sabex, Boucherville, QC, Canada). After a T7-9 laminectomy of the vertebral segment, 1 mm of spinal cord at Th8 was removed. In both groups the spinal cord stumps were then inserted into a 4 mm-long hollow guidance channel (2.6 mm inner diameter, 3.0 mm outer diameter; provided by T Hazlett, CytoTherapeutics, Providence, RI, USA), therefore ensuring lesion completeness. The untreated group received a 60:40 (v/v) solution of Dulbecco's modified Eagle's medium/F-12 in Matrigel. In treated rats, a suspension of 30  $\mu$ l of Schwann cells and olfactory ensheathing glia suspended in 60:40 (v/v) Dulbecco's modified Eagle's medium/F-12 in Matrigel were injected into the channel, the procedure differing from that used earlier, in which olfactory ensheathing glia were injected into the cord stumps.<sup>8</sup> Chondroitinase ABC (2  $\mu$ l of 10  $\mu$ g ml<sup>-1</sup>, cABC; Seikagaku America, East Falmouth, MA, USA) was administered as described earlier for a period of 4 weeks.<sup>8</sup> Uninjured animals ( $n=4$ ) were used as controls.

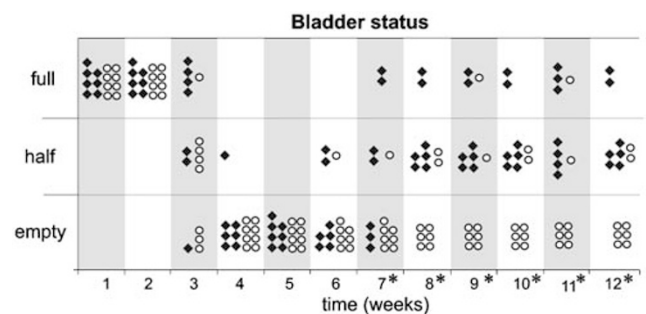
During the recovery period (12 weeks), injured-treated and untreated rats were group housed, mixed together in guinea-pig cages. Their bladders were manually emptied thrice per day for the first 3 weeks, then twice per day thereafter as required until voiding was re-established (that is, when there were only small amounts of residual urine left for manual expression) by personnel blind to the treatment of the rats. Bladder function was scored daily according to bladder filling: completely empty (no urine could be expressed),

moderately filled or full and whether the rat's perineum was wet or dry. Data from these notes presented in Figure 1 were chosen from every Wednesday of the week. For the first week post injury, rats received antibiotics (ampicillin sodium, 100 mg kg<sup>-1</sup>, Novopharm, Toronto, ON, Canada) twice per day. At 12 weeks, animals were killed with pentobarbital (Euthanyl, Bimeda-MTC, Cambridge, ON, Canada; 70 mg per 100 mg body weight) and transcardially perfused with 4% paraformaldehyde (0.1M, pH 7.4). Whole bladders were removed and emptied; the length (longitudinal—from the base/trigone to the apex) and the width (circumferential—at the widest part of the bladders) were measured to calculate the overall bladder size (in cm<sup>2</sup>). Bladders were then cut open and post fixed in formalin overnight, followed by 2 days in 30% sucrose phosphate-buffered saline.

Sections (36 mm<sup>2</sup>) of the lateral bladder wall (directly above the base) were removed from each bladder and frozen at 60 °C. Up to 20 consecutive transverse cryosections at 25  $\mu$ m were collected from each piece for histological analysis.

Bladder sections were stained with  $\alpha$ -smooth muscle actin (FITC conjugated, 1:200, Sigma, Oakville, ON, Canada) and collagen type III (1:200, Cedarlane Laboratories Ltd, Burlington, ON, Canada). Briefly, sections were dehydrated in an incubator at 37 °C for 1 h and washed twice for 10 min in 50 mM Tris-buffered saline (TBS), pH 7.4, followed by two 45 min washes with TBS containing 0.5% Triton X-100. Afterwards, the slides were incubated with a 10% normal goat serum in TBS (Vector Laboratories, Burlingame, CA, USA). This was followed by a 48-h incubation with primary antibody against  $\alpha$ -smooth muscle actin (at room temperature). After washing in TBS, slides were incubated for 24 h at room temperature in primary collagen type III antibody. For visualization of collagen, a Texas Red conjugated secondary antibody (1:200, Vector Laboratories) was used. The slides were dehydrated with alcohol and cleared with xylene and coverslipped in Permount (Fisher Scientific Ltd, Ottawa, ON, Canada).

Analysis of bladder wall thickness, actin and collagen type III staining intensity was carried out using a Leica DM6000



**Figure 1** Changes in bladder function from daily animal records. Notes from every wednesday of the post recovery period are shown. Directly following injury, both treated (white circles,  $n=8$ ) and untreated rats (black diamonds,  $n=7$ ) were unable to void. Both groups, recovered voiding within 4 weeks. Significant difference in bladder function developed after 7 weeks post injury as indicated by the asterisks.

fluorescent microscope (Leica Microsystems, Wetzlar, Germany). At  $\times 100$  magnification, the thickness of the bladder wall was measured using the Leica LAS AF 6000 software. Measurements of the maximal width of the bladder wall cross-sections were taken in five random sections. Then, digital images from three random sections per bladder were captured (Leica DFC350FX R2 digital camera, Leica Microsystems) and the levels of actin and collagen fluorescence were determined from a defined area spanning the entire diameter of the bladder wall using the histogram function in Adobe Photoshop. For the calculation of the collagen/actin ratio we used the median values of the measured fluorescence intensity.

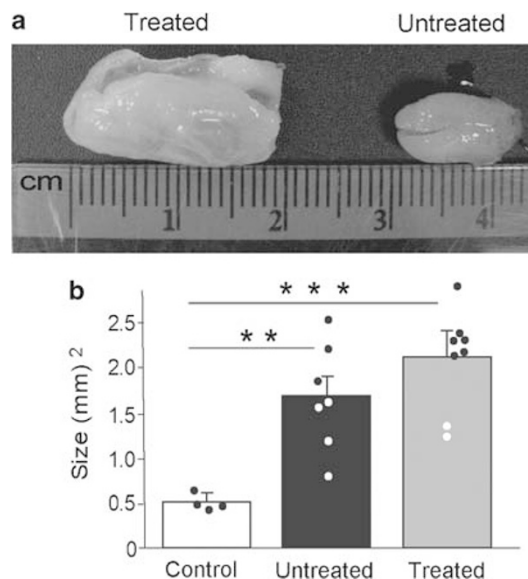
For the quantification of serotonergic fibers in the lumbar spinal cord, the cords were extracted following perfusion, post fixed in 4% formalin (with 5% sucrose) overnight, cryoprotected by storing tissue for 3 days in 30% sucrose, embedded in Tissue Tek (Sakura Finetek USA Inc, Torrance, CA, USA) and frozen in 2-methyl-butane over dry ice ( $-60^{\circ}\text{C}$ ). Then tissue was stored at  $-80^{\circ}\text{C}$  until further processing. For analysis, the lumbar spinal cord (L1-5) was sectioned into 25- $\mu\text{m}$  thick horizontal sections using a cryostat, mounted onto slides (Fisherbrand Color-Frost microscope slides, Fisher Scientific Ltd) and stored at  $-80^{\circ}\text{C}$  until staining procedure was performed. Every second section was stained for 5-HT with anti-serotonin primary antibody (1:1000, Sigma) overnight at  $4^{\circ}\text{C}$  followed by a biotinylated secondary antibody (1:200) and visualized with ABC/DAB method following the instructions of the Vector kit, SK4100, (Vector Laboratories). Afterwards, slides were washed in distilled water to halt the reaction and subsequently washed twice for 10 min in TBS. Sections were dehydrated in reduced alcohol concentrations, cleared with Xylene and coverslipped with Permount (Fisher Scientific Ltd).

For quantification of total fiber length, pictures of serotonin-positive fibers were taken at  $\times 400$  magnification using a Leica microscope system.

Statistical comparisons were performed using the Mann-Whitney test (GraphPad Prism, San Diego, CA, USA).

## Results

Immediately after SCI, all rats were unable to void and thus required manual bladder expression. Although the bladders were expressed thrice a day, a distension of the bladders in all animals was noticed during the phase of manual expression. During the first two postoperative weeks, three rats from the treated ( $n=8$ ) and two from the untreated group ( $n=7$ ) developed transient hematuria. Following 3 weeks, all rats exhibited spontaneous voiding, as their bladders were empty when manually expressed (Figure 1) and their lower abdominal and perineal areas were dry. Six animals that received the combined treatment (that is, 75%) maintained this status for the remainder of the testing period (that is, 12 weeks post injury). In contrast, in the remaining two treated rats and in 100% of the untreated rats the spontaneous voiding diminished after 2 weeks, a difference that was

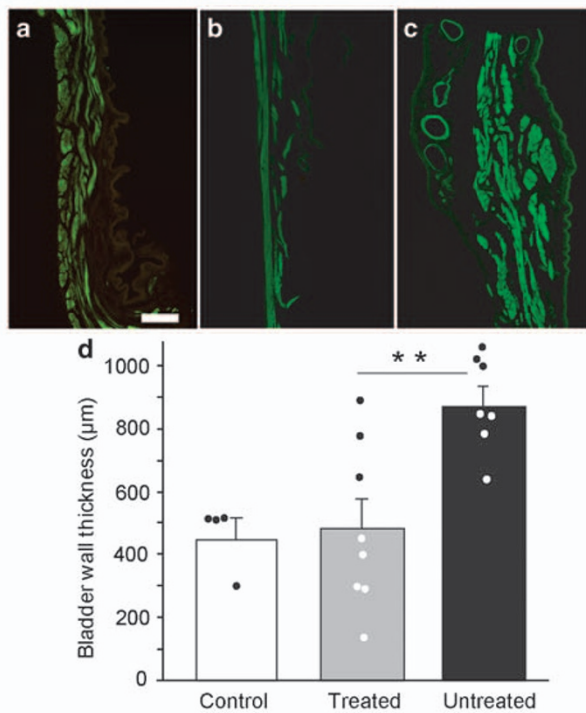


**Figure 2** Bladder sizes 12 weeks after complete spinal cord transection. (a) Representative images of treated and untreated bladder directly following perfusion illustrate thicker and stiff bladder walls, and a smaller size in the untreated group. (b) Quantification of bladder size revealed a significant injury-induced bladder increase; however, no statistical difference was seen between the treated and the untreated rats. Data are shown from single animals and as group representation (mean  $\pm$  s.e.m.). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

significant between the groups (Figure 1). When the bladders were palpated or manually expressed in these rats, we noticed a reduction in bladder size in comparison with the first 2 weeks post lesion. This was paralleled by the observation of constant urine leakage that kept their lower abdominal and perineal areas wet, although they retained small amounts of urine.

Following perfusion and subsequent measurement of bladder size, it was evident that the uninjured controls had significantly smaller bladders (area:  $0.54 \pm 0.07 \text{ cm}^2$ ,  $n=4$ ) than the injured rats with an area of  $1.69 \pm 0.21 \text{ cm}^2$  in untreated rats ( $P < 0.01$ ) and  $2.14 \pm 0.19 \text{ cm}^2$  ( $P < 0.001$ ) in the treated group. The difference in bladder size between the two injured groups was not statistically significant (Figure 2b). Bladders of untreated rats were also very stiff and exhibited thick, wrinkled bladder walls. These observations were quantified by measurements of the bladder wall thickness. We found significantly thicker bladder walls in untreated rats ( $881.2 \pm 57.6 \mu\text{m}$ ) when compared with treated animals ( $481.9 \pm 92.4 \mu\text{m}$ ) and uninjured controls ( $466 \pm 51.6 \mu\text{m}$ , Figure 3).

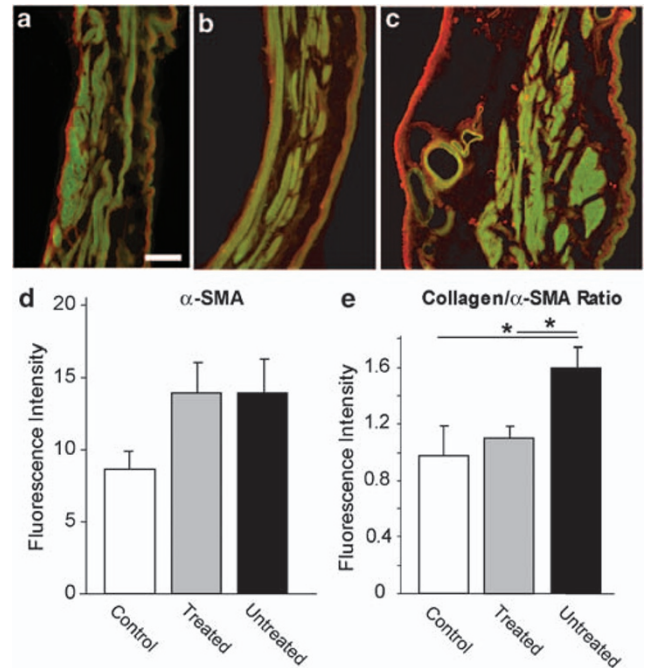
Immunohistochemical analysis of  $\alpha$ -smooth muscle actin in the cross-section of bladder wall tissue revealed an increase in thickness and disorganization of  $\alpha$ -smooth muscle bundles in the untreated group, whereas the treated group exhibited thinner, more organized and aligned  $\alpha$ -smooth muscle fiber composition (Figure 4). Although there seemed to be an increase in actin immunoreactivity in the treated and untreated group when compared with unlesioned controls, this difference was not statistically significant.



**Figure 3** Immunoreactivity for  $\alpha$ -smooth muscle actin in bladder wall sections from a unlesioned control (a), a treated (b) and an untreated (c) animal illustrate an increase in wall thickness in the untreated rats. (d) The measurements of bladder wall thickness ( $\mu\text{m}$ ) in control ( $n=4$ ), treated ( $n=8$ ) and untreated ( $n=7$ ) groups show a significant increase in bladder wall thickness in injured untreated rats. Scale bar =  $250\mu\text{m}$ .  $**P < 0.01$ . Data are shown from single animals and as group representation (mean  $\pm$  s.e.m.).

Although actin levels were comparable, similar analysis of collagen type III immunoreactivity showed significant accumulation of collagen in the bladders of untreated group when compared with treated animals after SCI. This is indicative of reduced viscoelasticity of the bladder walls in injured, untreated animals and is consistent with observed urine leakage, bladder stiffness and consequently the inability to store large volumes of urine. The ratio of collagen type III to  $\alpha$ -smooth actin in the bladders of the untreated SCI group ( $1.6 \pm 0.1$ ), an indication of the degree of significantly increased fibrosis, was higher in comparison with treated SCI rats ( $1.1 \pm 0.15$ ) or the bladders of unlesioned controls ( $0.97 \pm 0.2$ ; Figure 4).

As the restoration of caudal serotonergic innervation would seem a plausible mechanism for the improved bladder function in this study, we analyzed the total length of serotonin-positive fibers in the lumbar spinal cord of treated and untreated rats (Figure 5a). Against the common belief that following complete transection there are no serotonergic fibers in the cord caudal to the lesion site, we found short and few fibers in controls as well as treated rats. Fibers appear sparse and are fairly short (see below) with a beaded appearance, typical for serotonergic fibers. These findings confirm earlier observations from our laboratory and the



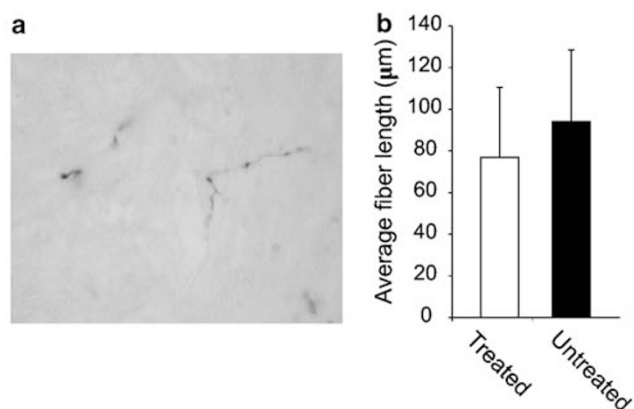
**Figure 4** Morphological changes in the bladder wall after SCI reveal increased collagen type III deposition. Collagen type III (red) and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA, green) immunofluorescence of an uninjured control (a), a lesioned-treated (b), and an untreated (c) bladder wall cross section. (d) Median levels of  $\alpha$ SMA in the bladder wall show only an insignificant increase in both injured groups. (e) Collagen type III/ $\alpha$ SMA ratios reveal a relational increase in collagen to  $\alpha$ SMA in injured untreated rats compared with both control and treated rats. Scale bar =  $250\mu\text{m}$ .  $*P < 0.05$ . Data are represented as group mean  $\pm$  s.e.m.

study by Newton and Hamill.<sup>11</sup> When analyzing the total length of these fibers, we did not find a statistical difference between control ( $94\mu\text{m} \pm 34.4$ ) and treated rats ( $77\mu\text{m} \pm 33.6$ ; Figure 5b).

## Discussion

Bladder management in patients with SCI struggles to reduce infections, preserve renal function and improve patient's quality of life. A primary factor that contributes to the frequent occurrence of infections is incomplete bladder voiding. Treatments that are able to provide persistent restoration or maintenance of bladder voiding following SCI could therefore substantially improve patient autonomy. Several studies with treatments designed to repair the injured spinal cord have reported to improve bladder function in animal models of SCI.<sup>12–14</sup> However, to date no studies have shown whether cell grafting can influence bladder function following a complete spinal cord transection. Experiments employing reparative approaches for complete SCI have largely focused on motor recovery, thereby neglecting to investigate potential therapeutic benefits of these strategies on bladder function.

At the conclusion of the current study, we found that untreated animals had developed a distinctly different



**Figure 5** Serotonergic fibers in the lumbar spinal cord. Short segments of serotonin-positive fibers with a beaded appearance (a) were found in the lumbar spinal cord of treated and untreated rats. When quantifying fiber length in every other section, the overall length of these fibers is not different between both groups (b).

bladder function than treated rats. In contrast to the dry perineal area of the treated rats, untreated animals were frequently wet indicating a constant urine leakage rather than distinct voiding periods. It could be argued that this outcome was influenced more by the difference in locomotor recovery than by changes in bladder function (for example, untreated rats are less mobile and stay more in one location). This seems, however, unlikely, as we showed earlier<sup>8</sup> that recovery in hind leg function is limited, and does not contribute significantly to the propulsion during locomotion. Furthermore, even if using only their forelimbs, rats are extremely mobile and can (depending on the traction) achieve surprising locomotor speed (unpublished observation). Lastly, the difference in voiding appeared after a phase during which both groups of rats showed comparable voiding, with rats in both groups being dry. Thus, it seems very unlikely that the progression to a different voiding pattern in the untreated group was caused by the inferior activity of these animals.

The finding that bladder size increased after the injury in the untreated group and the fact that SCI interferes with multiple aspects of lower urinary tract function is in accordance with an earlier study, in which a twofold increase in bladder size was shown 2 weeks following injury.<sup>15</sup> Our observations during bladder expression (during 2 weeks post lesion) indicated that bladder size increased initially in all animals during the initial weeks, however, it then decreased in the untreated animals. These findings in combination with the observed voiding efficacy in the two groups, suggests that it is not necessarily the increased bladder size that interferes with voiding and bladder dysfunction, but rather the secondary changes (that is, reduction in size). This idea is strengthened by the anatomical findings that untreated rats had bladders with a wrinkled appearance, with increased wall thickness, and whose walls contained a larger percentage of collagen type III. Augmented collagen type III deposition is considered an indicator of reduced bladder compliance and has been reported earlier both in

experimental animal models and humans with SCI.<sup>16,17</sup> This idea is supported by our observations of the untreated group, which showed reduced bladder capacity (that is, incomplete emptying of urine in addition to frequent urine expulsion). The reasons for the increase in bladder wall thickness and reduced compliance/increased collagen are unclear, but could be caused by bladder sphincter dysinergia causing continuous vesicular pressure that resulted in constant voiding/leakage.

The finding of extended bladders in injured-treated rats together with comparable wall thickness to the uninjured rats is somewhat counterintuitive. It possibly indicates that the overall amount of bladder tissue increased in the treated group. An outcome measure, which would have addressed this issue, could have been the weighing of the bladders. If the bladder tissue increase was due to an overall increase of actin in treated animals, it could be argued that this is the reason for a higher ratio of collagen to actin in the bladders of untreated rats. However, this seems unlikely, as the amount of actin in identical areas of the histological sections were statistically not different between treated rats and unlesioned animals. Thus, a possible overall increase in actin in treated rats would be due to the increase of the entire bladder size, but not due to the increase in wall cross-sections.

In our study, we report for the first time the maintenance of efficient bladder function after complete SCI in rats receiving a combination of cell grafts and scar-reducing enzyme, an approach for promoting axonal regeneration.<sup>8</sup> There are various possibilities that might explain the difference between the groups, including the re-establishment of descending circuitry or regeneration of neuromodulatory axons, including serotonergic fibers, as suggested earlier,<sup>18,19</sup> following a contusion injury<sup>13</sup> or a complete lesion.<sup>20</sup> The idea that serotonergic fibers might be involved in the observed maintenance of bladder function is supported by our earlier studies, in which serotonergic fibers, which had originated from the raphe nuclei, were able to regenerate across the cellular bridge and into the spinal cord caudal to the injury.<sup>8</sup> Furthermore, earlier literature showed that the injection of transneuronal tracer (Pseudorabies virus) into the external urethral sphincter or bladder labeled cells in the raphe magnus and raphe pallidus.<sup>3</sup> Lastly, it has been shown that stimulation of the raphe magnus and the gigantocellular reticular nucleus inhibits the spontaneous bladder contraction.<sup>21</sup> Thus, the restoration of caudal serotonergic innervation would seem a plausible mechanism for the improved bladder function in this study. However, the lacking difference in serotonergic innervation of the lumbar spinal cord found between treated and untreated rats suggest that other mechanisms were involved in the observed functional changes. With respect to our earlier finding of serotonergic fibers growing through and out of the graft,<sup>8</sup> the current results allow two conclusions. First, if there is any long distance regeneration of serotonergic fibers beyond the immediate caudal end of the graft and far into the lumbar spinal cord, it is insignificant, as the amount of fibers was comparable with that in untreated controls. Second, as the lumbar spinal cord of untreated controls

contained serotonergic fiber these must be of non-supraspinal origin as described earlier.<sup>11</sup>

An alternative mechanism for the maintenance of bladder function, that of re-established descending control, is supported by our earlier findings that following our combined treatment, axons originating from brainstem nuclei, such as the reticular formation as well as spinal interneurons, were regenerating across the lesion site.<sup>9</sup> As axons arising from the reticular formation modulate the activity of lumbar motoneurons either directly or through spinal interneurons, their regeneration could potentially be responsible for extending motoneurons survival, which in turn could maintain the efficient bladder function in the treated group.<sup>2</sup>

In summary, the current investigation expands the functional utility of our combined treatment and shows that cell transplantation and chondroitinase delivery can be beneficial for bladder function after complete SCI in rats. Unraveling the underlying mechanisms will offer a new basis for future studies focusing on the repair of the injured spinal cord and bladder rehabilitation.

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