

The effects of fetal allogeneic umbilical cord tissue transplant following experimental spinal cord injury on urinary bladder morphology

Wpływ alogenicznego przeszczepienia płodowej tkanki pępowiny na morfologię pęcherza moczowego w eksperymentalnym uszkodzeniu rdzenia kręgowego

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Abstract

Background and purpose: In continuation of our previous experimental study on spinal cord injury (SCI) using fetal stem cells, we investigated here the effects of fetal allogeneic umbilical cord tissue transplant on the urinary bladder morphology in a rat SCI model.

Material and methods: Five pregnant albino Wistar rats at 12 days of gestation were used to obtain the umbilical cord cell graft. In Group 1 ($n = 5$), Th8-Th9 laminectomy was performed. Group 2 ($n = 5$) received spinal cord injury. In Group 3 ($n = 5$), the cultured fetal umbilical cord cells coated with alginate gel were placed into the lesion cavity. In Group 4 ($n = 5$), only alginate sponges without umbilical cord cells were placed into the injury cavity. The bladders of animals were analyzed pathologically at 21 days after surgery.

Results: The thickness of the epithelium and the lamina propria did not differ among studied groups ($p > 0.05$). The lamina muscularis thickness was significantly higher in Group 2 and Group 4 than the others ($p < 0.05$). The bladder weight was similar among Groups 1, 2, and 3 ($p > 0.05$). Fibrosis was significantly increased in Group 2 ($p < 0.05$); it was greater in Group 2 than in Group 3 ($p < 0.05$) but did not differ between Groups 1 and 3 ($p > 0.05$).

Streszczenie

Wstęp i cel pracy: Kontynuując nasze wcześniejsze badania nad zastosowaniem płodowych komórek macierzystych w urazowym uszkodzeniu rdzenia kręgowego (*spinal cord injury* – SCI), przeprowadziliśmy badanie wpływu alogenicznego przeszczepienia płodowych komórek tkanek pępowiny na morfologię pęcherza moczowego w szczurzym modelu SCI.

Materiał i metody: Do uzyskania komórek pępowiny służących do przeszczepienia wykorzystano pięć ciężarnych samic białych szczurów rasy Wistar w 12. dniu ciąży. W grupie 1 ($n = 5$) wykonano laminectomię Th8-Th9. W grupie 2 ($n = 5$) dokonano urazowego uszkodzenia rdzenia kręgowego. W grupie 3 ($n = 5$) w miejscu uszkodzenia umieszczono hodowane komórki płodowe pępowiny powleczone żelem z alginianem. W grupie 4 ($n = 5$) do miejsca uszkodzenia podano wyłącznie gąbkę z alginianem bez komórek pępowinowych. Pęcherze moczowe zwierząt poddano ocenie patomorfologicznej po 21 dniach od zabiegu.

Wyniki: Grubość nabłonka i blaszki właściwej nie różniła się pomiędzy badanymi grupami ($p > 0,05$). Grubość mięśniówki błony śluzowej była istotnie większa w grupie 2 i 4 w porównaniu z pozostałymi ($p < 0,05$). Masa pęcherza była podobna w grupach 1, 2 i 3 ($p > 0,05$). Zwłóknienie pęche-

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Conclusions: This study suggests that allogeneic umbilical cord tissue transplantation after SCI may prevent bladder wall hypertrophy and fibrosis in the rat SCI model.

Key words: neurogenic, spinal cord injuries, stem cell transplantation, umbilical, urinary bladder.

Introduction

The normal filling and voiding functions of the lower urinary tract depend on a healthy and intact neural axis. Spinal cord injury (SCI) causes disruption of this mechanism, leading to important dysfunction of the lower urinary tract. The effect of SCI on voiding function is dependent on the level and completeness of injury [1,2]. The consequences of neuropathic bladder are renal failure due to a high bladder pressure, recurrent urinary tract infections, and urinary incontinence [2]. Urological treatments such as intermittent catheterization, anticholinergic medication, and augmentation cystoplasty are usually palliative for neuropathic bladder caused by SCI.

Rat models have been widely used to study the urological consequences of SCI [3]. In recent years, the role of transplantation of neural stem cells into the spinal cord has gained much attention for the treatment of SCI [4]. The beneficial effect of transplanted neural stem cells on voiding function in a rat model has been reported [5]. Stem cells derived from the umbilical cord blood, umbilical cord tissue, amniotic fluid and placenta have been used in different clinical and experimental studies [6-11]. Another potential source of mesenchymal cells is Wharton jelly of the human umbilical cord. Wharton jelly is the connective tissue surrounding the two arteries and one vein of the umbilical cord including fibroblast-like cells. It has been suggested that these cells are multipotent with the ability of proliferating and differentiating [6,10-12].

We have previously utilized a rat model of SCI to investigate the effects of fetal allogeneic umbilical cord tissue transplantation into spinal cord [13]. We demonstrated that fetal allogeneic umbilical cord cell transplantation improves motor function in spinal cord injured rats. In the present study, the effects of fetal allogeneic umbilical cord tissue transplant as a source of stem cells

was significantly greater in group 2 ($p < 0,05$); it was significantly greater in group 2 in comparison with group 3 ($p < 0,05$), but not significantly different between groups 1 and 3 ($p > 0,05$).

Wnioski: Wyniki badania wskazują, że alogeniczne przeszczepienie płodowej tkanki pępowiny w eksperymentalnym SCI może zapobiegać przerostowi i zwłóknieniu ściany pęcherza moczowego w szczurzym modelu SCI.

Słowa kluczowe: neurogeny, urazowe uszkodzenie rdzenia kręgowego, przeszczepienie komórek macierzystych, pępowinowy, pęcherz moczowy.

after spinal cord injury on the urinary bladder was tested in the same rats.

Material and methods

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee of Baskent University in accordance with the National Institute of Health for the care and use of laboratory animals.

Twenty adult male Wistar rats, aged 4-6 months, each weighing 268-342 g, were used in these experiments. The animals were anesthetized with intramuscular xylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg).

Preparation of fetal umbilical cord cells

Five pregnant albino Wistar rats at 12 days of gestation were used for obtaining the umbilical cord cell graft. Following the anesthesia, the pregnant rats were placed in the supine position. Fetuses were extracted from the uterus through a suprapubic incision. Only the umbilical cord was dissected and separated carefully using an operating microscope. Tissue samples were taken under sterile conditions. After the samples arrived at the laboratory, they were minced mechanically and tissue cultures were set up in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 200 μ M L-glutamine and penicillin/streptomycin in a 37°C CO₂ incubator. The cultures were harvested on the 10th day. Cultured cells were isolated by trypsin. Alginate gel was used as a carrier of grafted cells in this study. Sodium alginate or alginic acid sodium salt (Fluka, Switzerland) was used to prepare the alginate sponges. Alginate sponges were prepared by precipitation of the polymer in non-solvent (i.e. CaCl₂ solutions) in the form of a spongy type cylindrical rod. Alginate rods were sterilized

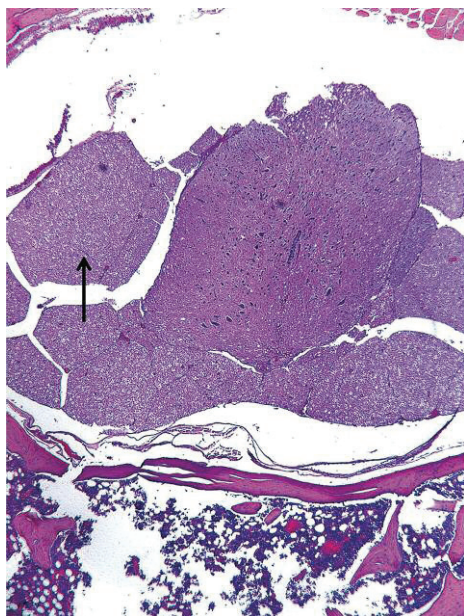


Fig. 1. Spinal cord section of the hemitranssected animals; black arrow indicates severe degenerative changes; shrunken cytoplasm and extensively dark pyknotic nucleus are seen in neuronal cells on the left side of the spinal cord. Hematoxylin and eosin, original magnification $\times 4$

with ethylene oxide and sterile three-dimensional alginate scaffolds that were minced into 1-2 mm sized fragments. The cell suspension (1 mL) was added to the tube and centrifuged. Alginate and cell mixtures were incubated in a 37°C CO₂ incubator overnight and the next morning the bound complexes were transplanted to the rats [13].

Experimental design

Group 1 (control group; $n = 5$): In this group, Th8-Th9 laminectomy was performed by a posterior midline approach. Care was taken not to damage the spinal cord in the control group.

Group 2 (trauma group; $n = 5$): Animals underwent only spinal cord injury, and received no treatment (hemitranssected animals). A wedge-shaped spinal cord hemitranssection by microscissors was performed after laminectomy. The microscissors were inserted into the spinal cord with the tip touching the midline until the left side of the cord was completely divided (Fig. 1).

Group 3 (transplantation group; $n = 5$): The cultured fetal umbilical cord cells which had been coated with alginate gel as a supporter for transplanted cells as described above were grafted into the lesion cavity immediately after the operation.

Group 4 (vehicle group; $n = 5$): Only alginate sponges without umbilical cord cells were placed into the injury cavity. After surgery, the wound was closed in layers.

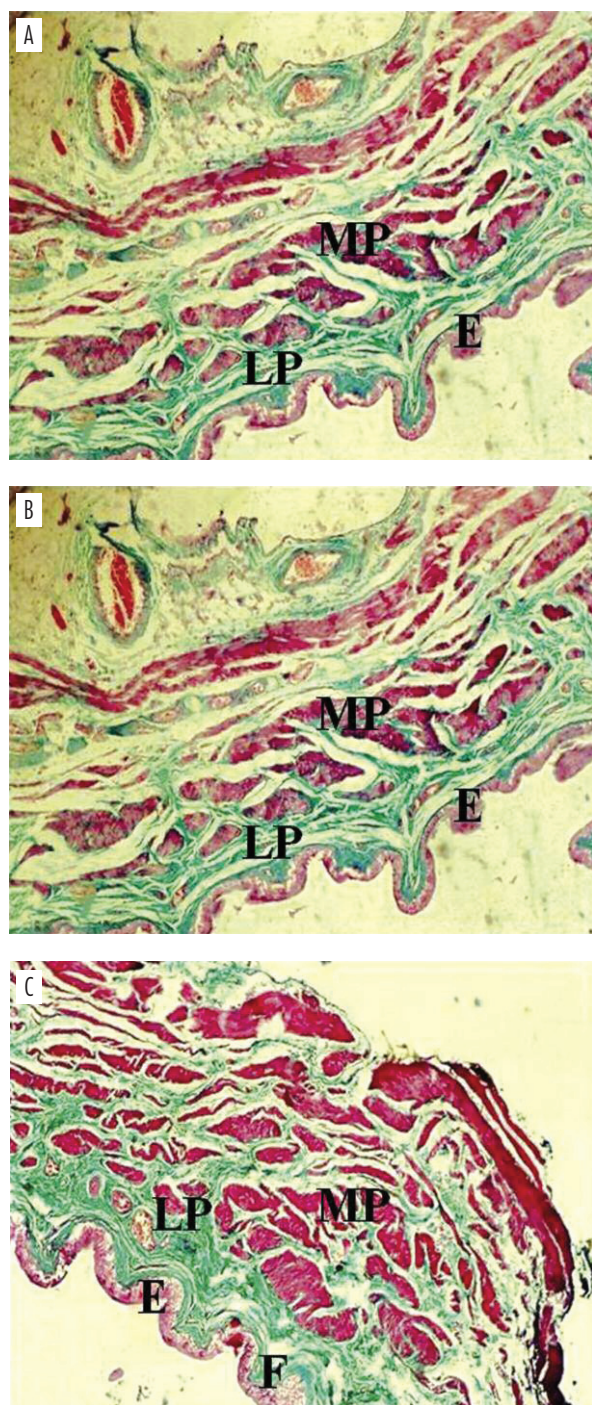
Pathological analysis of the urinary bladder was performed at the 21st day after surgery. At the end of each experiment period, the rats were sacrificed with an overdose of pentobarbital and then perfused transcardially with 50 ml of saline followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer.

Animal care

Rats were housed with free access to tap water and rat chow with a dark/light cycle of 12 by 12 hours. They were examined neurologically and the bladders were emptied by manual expression three times daily until reflex bladder emptying was established. All rats whether or not they received transplants were given 2.5 mg/kg subcutaneous enrofloxacin for 3 days and 10 mg/kg subcutaneous cyclosporine until the day of sacrifice to prevent rejection. No significant immune responses (graft rejection, etc.) such as inflammation and edema were detected microscopically in response to the transplanted material.

Light microscopy

The bladders of all rats were removed through a midline suprapubic incision. In addition to pathological analysis, recovery assessment was also indexed as the weight of the bladder. The bladders were weighed damp, using an electronic balance (Precisa XB 220A, Switzerland). For pathologic analysis, full thickness bladder samples were taken from the bladder dome. Five-micrometer thick, fixed paraffin-embedded tissue sections were stained with hematoxylin-eosin and Masson trichrome. All of the stained sections were evaluated by light microscopy by one pathologist who was blinded to tissue sample origin. The fibrotic changes, epithelium, lamina propria, and smooth muscle thickness were documented pathologically and compared in the four groups (Group 1, 2, 3, and 4). A calibrated eyepiece was used to measure the epithelium, lamina propria, and muscularis propria thickness on the sections stained with Masson trichrome at $\times 200$ magnification. On every slide, we measured the thickest part of the epithelium, lamina propria, and muscularis propria. Also, for each rat, two of the stained sections were randomly selected and the average epithelium, lamina propria, and muscularis propria thickness for these two sections was recorded. We used a new grading system for bladder fibrosis eval-



E – epithelium, F – fibrosis, LP – lamina propria, MP – muscularis propria

Fig. 2. Masson trichrome-stained bladder sections from rats: (A) Group 1 section demonstrates normal structure of bladder; (B) Group 2 bladder section demonstrates grade 2 fibrosis and muscular hypertrophy; (C) Group 3 section demonstrates grade 1 fibrosis. Original magnification $\times 40$

uation that is a modified form of the system defined by Testoni [14]. Extent of bladder fibrosis was graded according to the following classification: Grade 0 (none) – without fibrosis and muscular hypertrophy, normal structure of bladder (Fig. 2A); grade 1 (mild) – without muscular hypertrophy, only thin fibrotic tissue was observed between the epithelium and the lamina propria (Fig. 2C); grade 2 (severe): muscular hypertrophy and continuous fibrotic tissue was observed among the epithelium, the lamina propria, and the muscularis propria (Fig. 2B).

All of the results were recorded for statistical analysis.

Statistical analysis

Data were analyzed using SPSS for Windows (version 11.0; SPSS, Inc., Chicago, Illinois, USA). Data are expressed as mean $\pm$ standard deviation. Kruskal-Wallis test was used to compare the differences between groups, and the Mann-Whitney *U*-test was used for dual comparisons. In addition, bladder wall fibrosis was statistically analyzed using a standard χ^2 test. Differences were considered to be statistically significant when the probability value was less than 0.05.

Results

The epithelium thickness was higher in Group 4 (vehicle group) but the difference was not statistically significant among all groups ($p > 0.05$) (Fig. 3A). There were also no differences among groups for lamina propria thickness ($p > 0.05$) (Fig. 3B). The smooth muscle thickness was significantly greater in Group 2 (trauma group) and Group 4 (vehicle group) than the other two groups ($p < 0.05$) (Fig. 3C). In addition, a difference in the smooth muscle thickness was found among all groups ($p < 0.05$). The difference between Group 1 (control group) and Group 3 (transplantation group) was not significant ($p > 0.05$) but it was significant between Group 2 (trauma group) and Group 3 (transplantation group) ($p < 0.05$). The bladder weight differed among all groups ($p < 0.05$) (Fig. 4). There was no difference, however, among Group 1 (control group), Group 2 (trauma group), and Group 3 (Transplantation group) ($p > 0.05$). Table 1 shows the values of bladder wall fibrosis grades. When all groups were compared for fibrosis grades, a difference was found among all groups ($p < 0.05$). In addition, a difference was also found between Group 2 (trauma group) grades and Group 3 (transplantation group) grades ($p < 0.05$).

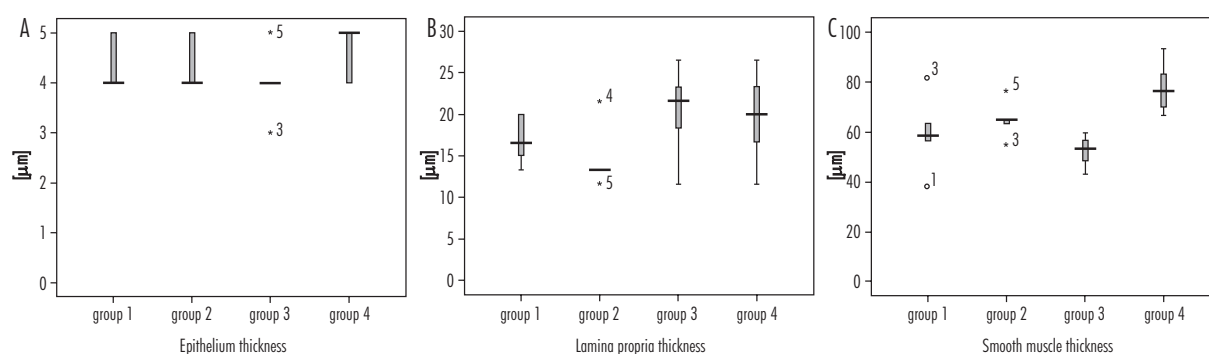


Fig. 3. Box-and-whisker plots demonstrating: (A) epithelium thickness of the sham and the experimental groups (similar among groups; $p > 0.05$) and standard deviations of the mean of epithelium thickness shown with vertical lines; (B) lamina propria thickness of the sham and the experimental groups (similar among groups $p > 0.05$) and standard deviations of the mean of lamina propria thickness shown with vertical lines; (C) smooth muscle thickness ($p < 0.05$ for the difference between Group 2 and Group 3; $p > 0.05$ for the difference between Group 1 and Group 3). Standard deviations of the mean of smooth muscle thickness are shown with vertical lines. Any outliers are marked with a circle and extreme cases with an asterisk. They are marked by their row number

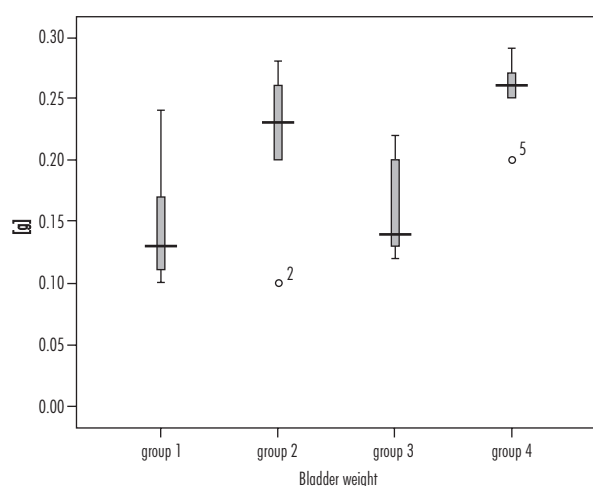


Fig. 4. Wet weight of bladder of the groups was determined in a box-and-whisker plot (Groups 1-3 were similar in this regard; $p > 0.05$). Standard deviations of the mean of bladder weight measurements are shown with vertical lines. Any outliers are marked with a circle. They are marked by their row number

but not between Group 1 (control group) grades and Group 3 (transplantation group) grades ($p > 0.05$).

Discussion

Spinal cord injuries (SCI) above the sacral segment can lead to severe urinary tract dysfunctions, including bladder areflexia, hyperreflexia, and detrusor-sphincter dyssynergy [15]. Also, bladder pathologies involve changes in tissue morphology such as hypertrophy [16] and fibrosis [17], as well as significant changes in mechanical properties [18]. Specifically, chronic neurogenic bladders tend to be less compliant than normal

Table 1. Bladder wall fibrosis values in the four groups

	Fibrosis		
	Grade 0	Grade 1	Grade 2
Group 1 ($n = 5$)	5	0	0
Group 2 ($n = 5$)	0	1	4
Group 3 ($n = 5$)	4	1	0
Group 4 ($n = 5$)	0	2	3

Definitions of fibrosis grades are provided in the text

bladders [17-19]. This loss of compliance appears to be related to changes in composition of collagen type I and III within the detrusor tissue, shown by increased ratios of collagen type III/collagen type I mRNA transcripts within the detrusor tissue [17].

Primary afferent and efferent components of storage and micturition reflexes are distributed bilaterally in spinal cord and overlap extensively. Similarly, both the sensory ascending tract and motor descending tract are important for synergic voiding [20,21]. Incomplete SCI result in an initial loss and later partial recovery of lower urinary tract function. In rats, urine release is mediated by contraction of the bladder detrusor accompanied by coordinated activation of the external urethral sphincter (EUS). Píkov *et al.* postulated that the presence of residual connections with brainstem control centers for micturition served as the anatomical basis of partial functional recovery [22]. They found that twenty percent white matter sparing at the injury epicenter was sufficient for complete recovery of detrusor-EUS coordination by 8 weeks after incomplete SCI. In the present study, a partial pathway lesion model was performed by unilateral excision of the spinal cord to have contin-

uous bladder function during surgery which led to survey of animals.

Different parts of the umbilical cord, which include cord blood, subendothelial layer of cord vein, cord vein endothelial lining and the cord Wharton jelly, contain stem cells with the ability of proliferating and differentiating into various tissues [6,8,10,11,23-25]. Umbilical cord blood stem cells have demonstrated efficacy in reducing lesion sizes and enhancing behavioral recovery in animal models of ischaemic and traumatic central nervous system injury [25]. Wang *et al.* [26] transplanted human umbilical cord mesenchymal stem cells (hUCMSCs) into hemitranssected spinal cord in a murine model and noted that transplanted hUCMSCs survived and migrated to the injured site. Non-embryonic stem cells has already begun to be utilized to treat a variety of diseases including cardiovascular, hepatic, ophthalmic, orthopedic, neurological and endocrine diseases [27].

The purpose of this study was to evaluate the effect of allogeneic umbilical cord cell transplantation on the pathological changes in the bladder caused by incomplete SCI. There were no statistically significant differences between the groups in epithelium thickness and lamina propria thickness. However, lamina muscularis thickness and fibrosis grades were found significantly increased in Group 2 (trauma group) and Group 4 (vehicle group) compared to the others. The epithelium thickness was also highest in Group 4 (vehicle group).

Sakamoto and coworkers investigated the extent of bladder changes in bladder structure after treatment with neurotrophin-secreting Schwann cells and carbon filament implantation following severe spinal cord contusion [28]. Two months after injury, they performed cystometry and studied the bladders using light microscopy. The untreated SCI bladders underwent a large increase in bladder wall thickness, primarily due to an increase in smooth muscle and urothelium. Treated bladders had bladder wall, urothelium and smooth muscle thicknesses greater than controls but less than untreated SCI bladders. They concluded that neurotrophin-secreting Schwann cell implants may help to improve bladder structure after spinal cord injury. In the present study, increased bladder muscle measures were found in Group 2 (trauma group). These results are compatible with the findings of Sakamoto *et al.* In addition, in Group 3 (transplantation group) the bladder muscle measurement was found similar to Group 1 (control group). Furthermore, the bladder weight measurements were found to be higher in Group 2 (trauma group) than Group 3 (transplantation group). These

results showed that animals with increased muscle measurements had increased weight of bladders.

Previously, it was reported that inflammation is a central driver of the physiology of people with SCI, and it is apparent that inflammation has both beneficial and detrimental functions [29-31]. The bladder appears to be one of the organs that suffer from these systemic effects. Deveaud *et al.* [17] demonstrated that both lymphatic nodules and diffuse aggregates of lymphocytes were present in the lamina propria layers of many bladder tissue specimens, indicating inflammation of the tissue. In addition, it was also shown that the inflammatory cell infiltrate had been located solely in the lamina propria, together with fibrosis [32]. Our present study supports these previous reports because in Group 2 (trauma group) high fibrosis grades were found. These results may be related to direct injury to the spinal cord.

This study supports the results of our previous study that umbilical cord cell transplantation has beneficial effects on SCI. Our findings are encouraging in that we have found some pathological improvement as described above. More experiments will be required to determine whether the transplantation of umbilical cord tissue cells as a source of stem cells into the completely divided (transection) rat spinal cord would produce functional benefits for the urinary bladder proved by urodynamic studies.

Conclusions

1. Results of this study together with those of our previous study suggests that allogeneic umbilical cord cell transplantation after SCI has beneficial effects on the spinal cord and may prevent urinary bladder wall hypertrophy and fibrosis.
2. Further studies are needed to investigate the effects of allograft transplantation on urodynamic parameters and molecular modulators of pathologic smooth muscle hypertrophy and fibrosis in spinal cord injury subjects.

Disclosure

Authors report no conflict of interest.

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