

Histopathological effects of mesenchymal stem cells in rats with bladder and posterior urethral injuries

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Background/aim: To evaluate the effects of mesenchymal stem cell (MSC) therapy in an experimental bladder and posterior urethral injury model.

Materials and methods: The study subjects consisted of 40 male Wistar albino rats that were divided into four groups: control group (n = 10) (the bladder was only surgically opened and closed), sham group (n = 10) (surgical procedure), IVMSC group (n = 10) (surgical procedure and intravenous MSC treatment), and LMSC group (n = 10) (surgical procedure and local MSC treatment). Histopathological evaluation was performed for the degree of fibrosis and inflammation and the extent and intensity of staining of vascular endothelial growth factor (VEGF) and endoglin (CD105).

Results: There were no significant differences between the control and LMSC groups with respect to fibrosis (P = 0.070) or inflammation (P = 0.048). Fibrosis and inflammation were significantly lower in the IVMSC (P = 0.034 for fibrosis, P = 0.080 for inflammation) and LMSC (P = 0.01 for fibrosis, P = 0.013 for inflammation) groups when compared with the sham group. No significant differences regarding fibrosis and inflammation were observed between the IVMSC and LMSC groups (P = 0.198 for fibrosis, P = 0.248 for inflammation). A significant difference was noted between the sham and LMSC groups concerning VEGF staining intensity (P = 0.017). However, no significant difference was found among the groups with regard to the extent or intensity of CD105 staining (P > 0.05).

Conclusion: MSC treatment significantly decreased the development of fibrosis in a uroepithelial injury model.

Key words: Urinary bladder, urethra, mesenchymal stem cell, posterior urethral stricture, fibrosis

1. Introduction

Although bladder and posterior urethral injuries are not usually life-threatening conditions in children, they may cause long-term morbidity that seriously affects patient quality of life. Serious complications may occur after bladder and posterior urethral injury including urinary incontinence, urethral stricture, impotence, and a need for intermittent bladder catheterization (1–3). The cause of the complications may depend on the injury itself, as well as the operations performed during the treatment. The main problem is fibrosis developing during wound healing. If the degree of fibrosis is reduced, many complications can be avoided.

Mesenchymal stem cells (MSCs) can regenerate and differentiate into different cell and tissue types. MSCs reach damaged tissue, after which they regenerate and differentiate into the involved tissue type to promote the maintenance of function (4,5). A great number

of stem cell studies have yielded promising results in experimental disease models (6–8). MSC therapy had been studied in the treatment of urological diseases like stress urinary incontinence, bladder dysfunction, and erectile dysfunction. Their results are quite successful (9). Wang et al. showed in their experimental study on rabbits that combined fibrin glue and MSC treatment in repairing urethral injury may improve neovascularization and smooth muscle formation (10). We did not find a study evaluating the histopathologic effects of mesenchymal stem cell therapy on uroepithelial wound healing.

In our study, we evaluated the effect of MSCs in an experimental rat model of bladder and posterior urethral injury.

2. Materials and methods

The current study was approved by the Animal Experimentation Ethics Committee of Ondokuz Mayıs

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University (approval no. 2011/54). All surgical procedures were performed at the Research Center for Animal Experiments at Ondokuz Mayıs University. All animals were individually caged in a room under standard environmental conditions and were fed a standard rat diet.

2.1. MSC isolation

Male rats were used as donors to isolate MSCs. They were euthanized by ether inhalation. The medullary cavities were flushed with Dulbecco's modified Eagle's medium/low glucose (DMEM-LG). Bone marrow was extracted from the medullary cavities of each rat's femur, tibia, and humerus using a washing method. Cells suspended in DMEM-LG were added to tubes over an equal volume of Ficoll solution. To separate the mononuclear cells, the tubes were centrifuged at 900 rpm for 30 min. After mononuclear cells were removed from the interface, the cells were seeded at a concentration of $1 \times 10^6/\text{mL}$ in 25-cm^2 (T25) cell culture flasks and suspended in DMEM-LG containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 Ug/mL streptomycin. Cells were incubated at 37°C and 95% humidity in an atmosphere of 50 mL/L CO_2 . The medium was changed daily during the first 3 days and once every 3 days thereafter. Nonadherent cells were discarded. Cells were collected at 80%–90% confluence and amplified afterwards. After passages, the bone marrow cells became homogeneous. MSCs were stored at -80°C . The cells, suspended at the time of use, were prepared in 1-mL insulin injectors at a concentration of 2×10^6 cells/mL for use in subjects.

2.2. Experimental groups and surgical technique

Forty male Wistar albino rats weighing 280–300 g were used. The animals were divided into four groups. After 8 h

of fasting, the rats were injected with a mixture of 80 mg/kg ketamine HCl (Ketalar, Pfizer, Turkey) and 12 mg/kg xylazine (Rompun, Bayer, Turkey) intraperitoneally and then they were anesthetized. Preoperative intramuscular 50 mg/kg ceftriaxone (Forsef, Bilim, Turkey) prophylaxis was administered. Povidone-iodine sterilization was performed before each procedure. At the beginning of this study, we did preliminary work in order to determine the appropriate surgical procedure. This was an original procedure for bladder and posterior urethra injury. Laparotomy was performed through a suprapubic vertical incision. The bladder was opened via an approximately 1-cm-wide incision. Triangle-shaped tissue (length of each side, 0.5 cm; depth, $<1\text{ mm}$) was sectioned from the posterior bladder wall extending from the bladder trigone to the posterior urethra (Figure 1).

In the control group ($n = 10$), no intervention was performed. After the surgical procedure in the sham group ($n = 10$), MSC treatment was not applied. In the IVMSC group ($n = 10$), MSCs were injected through the caudal vein. After the surgical procedure in the LMSC group ($n = 10$), MSCs were injected locally at the defected tissue site intramuscularly (Figure 2).

2.3. Histopathological examination

The rats were sacrificed after 21 days. The specimens were suspended in 10% buffered neutral formol for 24 h before histological examination. The specimens with surgical injuries were embedded in paraffin, and $5\text{-}\mu\text{m}$ -thick slices were obtained. Tissue slides were stained with hematoxylin-eosin and Masson's trichrome for histological examination and immunohistochemical staining was performed using vascular endothelial growth factor (VEGF) monoclonal

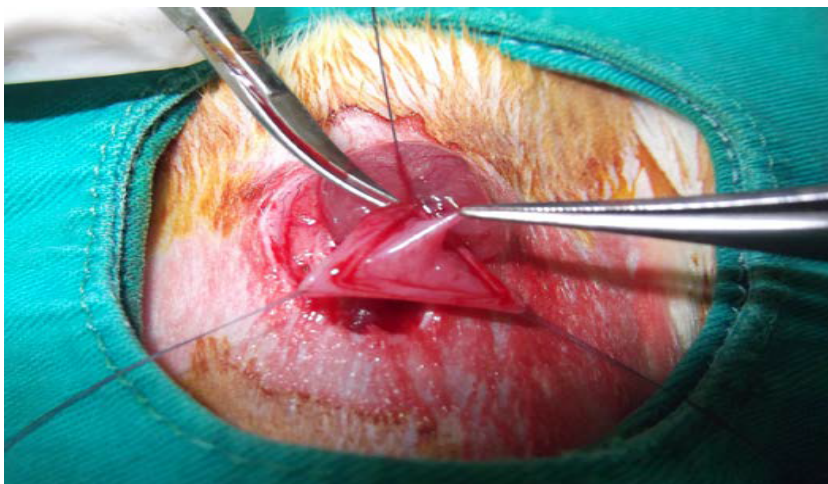


Figure 1. Bladder and posterior urethra injury model (triangle-shaped tissue was sectioned from the posterior bladder wall extending from the bladder trigone to the posterior urethra) (original).

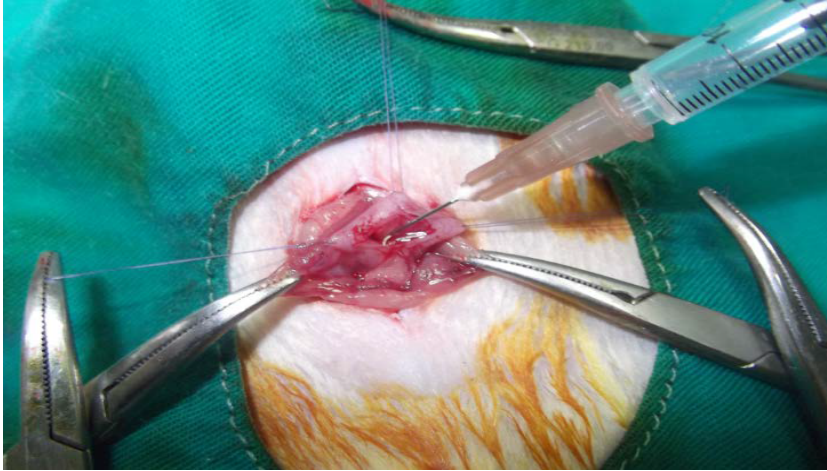


Figure 2. Mesenchymal stem cells were injected locally at the defected tissue site intramuscularly (original).

antibody (Thermo Scientific, USA) and endoglin (CD105) (Thermo Scientific, USA). Cytoplasmic staining was also evaluated in the immunohistochemical examination.

Inflammation (lymphocytic infiltration) and fibrosis were scored as follows: 0, absence of fibrosis/inflammation; 1, mild fibrosis/inflammation (<25%); 2, moderate fibrosis/inflammation (between 25% and 50%) and 3, value indicated severe fibrosis/inflammation (>50%) (Table 1) (11).

A scoring system was developed to evaluate VEGF and CD105 staining in fibroblasts. The extent and intensity of staining in fibroblasts were evaluated for VEGF and CD105. The extent of staining was scored as follows: 0,

less than 10%; 1, between 10% and 50%; and 2, $\geq 50\%$. For the staining intensity, no staining, yellow staining, brown staining, and dark brown staining were scored as 0, 1, 2, and 3, respectively (Table 2) (12).

2.4. Statistical analysis

SPSS 13.0 was used for statistical analysis. The chi-square or Fisher's exact test was used for statistical analysis. A P value ≤ 0.05 indicated statistical significance.

3. Results

The intensity of fibrosis is shown in Figure 3. Only one case of mild fibrosis was found in the control group. In the sham group, all specimens displayed fibrosis (one, six, and two

Table 1. Fibrosis and inflammation assessment score.

Absent fibrosis/inflammation	no	0
Mild fibrosis/inflammation	<25%	1
Moderate fibrosis/inflammation	25%–50%	2
Severe fibrosis/inflammation	>50%	3

Table 2. Vascular endothelial growth factor (VEGF) and endoglin (CD105) staining score.

Staining extent	<10%	0
	10%–50%	1
	>50%	2
Staining intensity	No staining	0
	Yellow staining	1
	Brown staining	2
	Dark brown staining	3

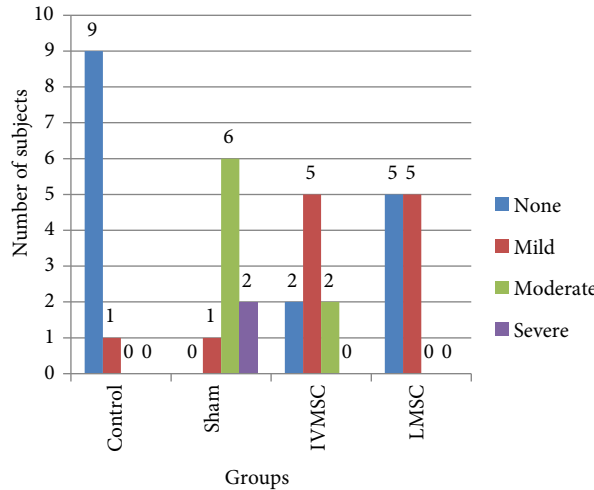


Figure 3. Intensity of fibrosis in the groups (IVMSC group: intravenous mesenchymal stem cell treatment group, LMSC group: local (intramuscular) mesenchymal stem cell treatment group).

cases of mild, moderate, and severe fibrosis, respectively). In the IVMSC group, mild and moderate fibrosis were detected in five and two specimens, respectively. In the LMSC group, five specimens exhibited no fibrosis, whereas the remaining five specimens displayed mild fibrosis. In terms of the development of fibrosis, moderate or severe fibrosis was observed in more than half of the specimens in the sham group, whereas moderate or severe fibrosis was not detected in the LMSC group. Meanwhile, no fibrosis was observed in two specimens of the IVMSC group. There were no significant differences between the control and LMSC groups with respect to fibrosis ($P = 0.070$).

Fibrosis was significantly lower in the IVMSC ($P = 0.034$) and LMSC ($P = 0.01$) groups when compared with the sham group. No significant differences regarding fibrosis were observed between the IVMSC and LMSC groups ($P = 0.198$). Statistical analysis of fibrosis scores and average scores of the groups is presented in Table 3.

The intensity of inflammation is shown in Figure 4. In the control group, mild inflammation was found in two specimens. In the sham group, two, five, and two cases of mild, moderate, and severe inflammation, respectively, were detected. In the IVMSC group, five and two specimens displayed mild and moderate inflammation, respectively,

Table 3. Fibrosis scoring P values and average values of groups (IVMSC group: intravenous mesenchymal stem cell treatment group, LMSC group: local (intramuscular) mesenchymal stem cell treatment group).

Groups	Group average value	Contrastive P value	
Control	0.1	Sham	-
		IVMSC	0.011
		LMSC	0.070
Sham	2.1	Control	-
		IVMSC	0.034
		LMSC	0.001
IVMSC	1	Control	0.011
		Sham	0.034
		LMSC	0.198
LMSC	0.5	Control	0.070
		Sham	0.001
		IVMSC	0.198

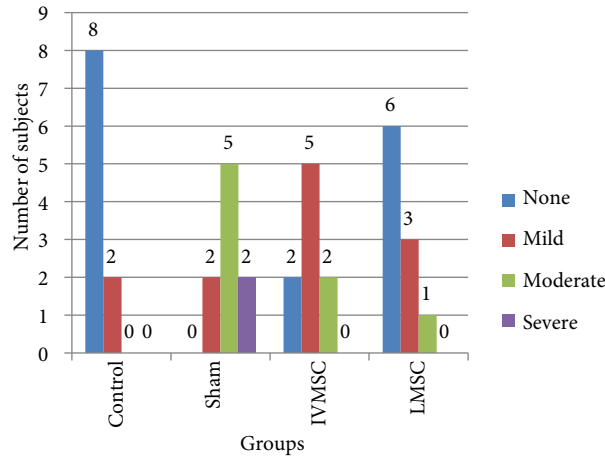


Figure 4. Intensity of inflammation in the groups (IVMSC group: intravenous mesenchymal stem cell treatment group, LMSC group: local (intramuscular) mesenchymal stem cell treatment group).

whereas two specimens exhibited no inflammation. In the LMSC group, three and one specimen displayed mild and moderate inflammation, respectively, whereas six specimens had no inflammation. There were no significant differences between the control and LMSC groups with respect to inflammation ($P = 0.048$). Inflammation was significantly lower in the IVMSC ($P = 0.080$) and LMSC ($P = 0.013$) groups when compared with the sham group. No significant differences regarding inflammation were observed between the IVMSC and LMSC groups ($P = 0.248$). Statistical analysis of the inflammation scores and average scores of the groups is presented in Table 4.

In the control group, the extent of VEGF staining was scored as 0, 1, and 2 in four, three, and two specimens, respectively, and the intensity score was 2 for all specimens. In the sham group, the extent of VEGF was scored as 0, 1, and 2 in one, three, and five specimens, respectively. The intensity scores were 1 and 2 in three and five specimens, respectively. In the IVMSC group, the extent of VEGF staining was scored as 0, 1, and 2 in one, four, and one specimen, respectively, and the intensity was scored as 2 in all specimens. In the LMSC group, the extent of VEGF staining was scored as 0 and 1 in five specimens each, and the intensity was scored as 2 for all specimens.

Table 4. Inflammation scoring P values and average values of groups (IVMSC group: intravenous mesenchymal stem cell treatment group, LMSC group: local (intramuscular) mesenchymal stem cell treatment group).

Groups	Group average value	Contrastive P value	
Control	0.2	Sham	-
		IVMSC	0.033
		LMSC	0.48
Sham	2	Control	-
		IVMSC	0.080
		LMSC	0.013
IVMSC	1	Control	0.033
		Sham	0.080
		LMSC	0.248
LMSC	0.5	Control	0.48
		Sham	0.013
		IVMSC	0.248

The difference between the sham and LMSC groups was statistically significant ($P = 0.017$), whereas no statistically significant differences were observed between the other groups. When the extent and intensity of staining were evaluated together, no significant differences were found among the groups ($P > 0.05$).

The extent of CD105 staining in the control group was scored as 0 and 1 in six and three specimens, respectively, and the intensity was scored as 2 for all specimens. In the sham group, the extent of CD105 staining was scored as 0, 1, and 2 in one, seven, and one specimen, respectively, and the intensity was scored as 1 and 2 in one and eight specimens, respectively. In the IVMSC group, the extent of CD105 staining was scored as 0, 1 and 2 in one, four and one specimen, respectively, and the intensity was scored as 2 in all specimens. In the LMSC group, the extent of CD105 staining was scored as 0 and 1 in three and six specimens, respectively, and the intensity was scored as 2 in all specimens. Evaluating the extent and intensity of CD105 staining separately or together, no differences were found among the groups ($P > 0.05$).

4. Discussion

Bladder and posterior urethral injury in children represents severe trauma with high morbidity rates including urinary incontinence, urethral stricture, multiple operations, and a lifetime requirement for intermittent catheterization (3,13). The options for managing bladder and posterior urethral injuries are conservative treatment, immediate primary intervention, and intervention after the immediate period (14). However, there is no agreement regarding the standard care of these injuries in the literature (15,16). Eliot et al. reported that after primary immediate urethral realignment of 53 patients with posterior urethral injuries 11 (21%) had erectile dysfunction, 2 (3.7%) had urinary incontinence, 36 (68%) had anastomotic strictures, and 13 (24.5%) had significant strictures requiring a repeat procedure in their 11 years of follow-up (17). However, Webster et al. stated that the rate of urethral stricture was 10% after late-term treatment in his clinical series (18). Mouraviev et al. mentioned that patients who undergo early realignment have lower incidences of erectile dysfunction and urinary incontinence than those who undergo delayed open urethroplasty (19).

Urethral stricture caused by the development of fibrosis secondary to surgical intervention may cause serious problems during long-term follow-ups. Incontinence may be associated with the severity of trauma, or it may develop during the management process for urethral strictures. Although some authors aim to decrease fibrosis via early interventions, some argue that better results are achieved with late interventions (3,20). Treatment is a challenging process in patients with bladder and posterior urethral

injuries because its consequences are variable. Previous clinical series indicate that complications are less frequent if fibrosis is less severe (21,22). Thus, decreasing the rates of fibrosis and stricture can prevent a large number of future complications. Preventing the development of fibrosis appears more reasonable than many of the treatment measures performed after fibrosis develops. Accordingly, we conducted this study aiming to develop an alternative to available treatment modalities for preventing the development of fibrosis during uroepithelial recovery.

MSCs are nonhematopoietic multipotent cells with the ability to differentiate into both mesenchymal and nonmesenchymal cells. These cells can be obtained by bone marrow by aspiration, and they do not require immunosuppression during the treatment course due to their low immunogenicity. Thus they are good candidates for experimental research (23). When MSC reproduction can be controlled, it is possible to differentiate the cells into the necessary cell types in *in vitro* laboratories. MSC treatments for heart muscle regeneration, degenerative bone diseases, diabetes mellitus, atherosclerotic blood vein regeneration, and central nervous system diseases, such as Alzheimer disease, Parkinson disease, and spinal injuries, have become more promising (24,25).

MSC treatment may have been successful in various experimental studies because it decreased serious morbidity caused by extensive fibrosis in models of bladder and posterior urethral injury. Our results indicate that MSC treatment is effective for reducing fibrosis. In the current study, although the sham group animals exhibited spontaneous recovery, more than half of the subjects had moderate fibrosis, and 25% had severe fibrosis and inflammation. In contrast, severe fibrosis or inflammation was not observed in the groups that received IVMSC or LMSC treatment.

An experimental study involving dogs indicated that MSCs applied intradermally to skin wounds have positive effects on healing (26). In an intestinal ischemic reperfusion model in rats, submucosal application of MSCs has been reported to significantly reduce damage in the villi of the mucosa compared with the control (27). Similarly, MSC application to the surgical site in subjects with ischemic left colon anastomosis has been shown to improve results and positive effects on tissue healing (28). In our study, no significant differences were found between the control and LMSC groups with respect to the development of fibrosis and inflammation. This result indicates that the local application of MSCs improves the healing process, making it comparable to intact tissue.

Zickri et al. compared the possible effect of intraperitoneal stem cell therapy on lung injury with that of intravenous stem cell therapy and found that the effect of intraperitoneal MSCs was slightly inferior to that of

intravenous MSCs (29). Our study, featuring two different strategies of MSC application (local and intravenous), provided additional data regarding the efficacy of different delivery methods for MSC treatment. Although the improvement of fibrosis and inflammation in the LMSC group is not statistically significant, it is better than that in the IVMSC group and if this treatment is given locally it can be more effective.

The results for inflammation were in line with those for fibrosis, and they show that MSCs have positive healing effects on inflammation. MSCs promote VEGF expression and they express CD105. CD105 is a marker that is positive in 95% of MSCs, and its presence is confirmed while isolating MSCs. VEGF and CD105 are regarded as markers of angiogenesis during wound healing. We evaluated VEGF and CD105 staining in all groups, but there was no significant difference among the groups. In the groups treated with MSCs, although fibrosis and inflammation scores were lower, mild staining for CD105 and VEGF may indicate the potentially positive effects of MSCs on wound healing through these cytokines. Many

studies have demonstrated that MSCs stimulate local stem cell sources by inducing cytokine-mediated effect in tissues such as the heart, neurons, and pancreas that do not have spontaneous proliferation ability (30,31). We suggest that VEGF and CD105 are not appropriate markers for evaluating healing in damaged cells due to the confounding effects of coexisting inflammation.

In the current study, the effects of MSCs in damaged areas were assessed using the marked MSC or analyzing the SRY gene. To demonstrate the wound-healing effects of MSCs, different markers could be used.

In conclusion, the optimal management strategies for bladder and posterior urethral injuries have been debated for years. MSC treatment may reduce and even prevent fibrosis and inflammation, which are regarded as causes of remarkable morbidity. It appears that the application of MSCs, particularly LMSC treatment, can effectively reduce fibrosis. Further studies are needed to confirm the positive effects of MSCs on bladder and urethral wound healing by decreasing fibrosis and inflammation.

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