

EFFECT OF PERIURETHRAL INJECTION ADIPOSE-DERIVED STEM CELLS IN NERVE HEALING AND VOIDING BEHAVIOR FOR THE TREATMENT OF STRESS URINARY INCONTINENCE IN A RAT MODEL

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ABSTRACT

Objective: To investigate whether ADSCs are capable of nerve repair in stress urinary incontinence. **Material & Methods:** Thirty rats were allocated to five groups for receiving a periurethral injection with Phosphate Buffer Saline (PBS) (B1, B2) and Adipose-derived Stem Cells (C1, C2). Functional assessments with Voided Stain on Paper (VSOP) Method were reassessed at 2 and 4 weeks after injection. The rat SUI model was generated by Vaginal Distention (VD). The regeneration of peripheral nerve was evaluated by PGP 9.5 immunohistochemical staining. **Results:** Results revealed significant peripheral nerve differences between ADSC group and other groups in 28 days using PGP 9.5 staining. Our functional assessment, Voided Stain on Paper (VSOP), showed that ADSC group had significant improvement in 14 days; unfortunately, our 28 days improvement was not substantial. **Conclusion:** Periurethral injection of ADSCs improves urine pattern in SUI-induced rats and increase the amount of peripheral nerve. This method may be a potential strategy for SUI treatment.

Keywords: Vaginal Distention, Adipose-derived stem cells, PGP 9.5, Nerve healing periurethral, voided stain on paper.

ABSTRAK

Tujuan: Mengetahui apakah ADSCs mampu memperbaiki saraf pada SUI. **Bahan & Cara:** Tiga puluh tikus dialokasikan ke lima kelompok untuk menerima injeksi periuretral dengan Phosphate Buffer Saline (PBS) dan Adipose-derived Stem Cells. Penilaian fungsional dengan Metode Voided Stain on Paper (VSOP) dinilai kembali pada 2 dan 4 minggu setelah injeksi. Model SUI tikus dihasilkan oleh Vaginal Distention (VD). Regenerasi saraf tepi dievaluasi dengan pewarnaan imunohistokimia PGP 9.5. **Hasil:** Hasil menunjukkan perbedaan saraf tepi yang signifikan antara kelompok ADSC dan kelompok lain dalam 28 hari. Penilaian fungsional kami, Voided Stain on Paper (VSOP), menunjukkan bahwa kelompok ADSC mengalami peningkatan yang signifikan dalam 14 hari. Sayangnya, peningkatan 28 hari kami tidak signifikan. **Simpulan:** Injeksi ADSCs periurethral memperbaiki pola urin pada tikus yang diinduksi SUI dan meningkatkan jumlah saraf tepi. Metode ini mungkin merupakan strategi potensial untuk pengobatan SUI.

Kata Kunci: Vaginal Distention, Adipose-derived stem cells, PGP 9.5, penyembuhan saraf periuretral, voided stain on paper.

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INTRODUCTION

Stress urinary incontinence (SUI) is a common problem experienced by many women. SUI can have a significant negative impact on the quality of life (QOL) of not only those who suffer from the condition, but also potentially on those friends and

family members whose lives and activities may also be limited.¹ Approximately 49% of all urinary incontinent women suffer from stress urinary incontinence (SUI), 29% from mixed incontinence, and 22% from urge incontinence.² The prevalence of daily incontinence increased with age, ranging from 12.2% in women 60 to 64 years old to 20.9% in

women 85 years old or older.³ Urinary incontinence causes depression, anxiety, poor life satisfaction, and impaired quality of life.⁴⁻⁶ The pathophysiology of SUI is multifactorial, including atrophy of the smooth muscle and the rhabdosphincter, changes in connective tissue, changes in blood perfusion in periurethral vasculature, submucosal tissue, and neuronal mass. Those changes are seen after vaginal delivery.⁷ The therapeutic modalities for SUI still consist of non-surgical and surgical procedures. Many publications support that this treatment was ineffective in some patients and may lead to side effects.⁸

Vaginal delivery of children causes traumatic injury to tissues of pelvic floor. It is strongly correlated with later development of SUI.⁹ Multiple animal models have been used to study the effect of stem cells in tissue regeneration of the urethra. Many experimental models that simulate some of the components of the human pathophysiology of SUI due to trauma of vaginal delivery, such as vaginal balloon dilatation¹⁰, pudendal crush injuries¹¹, sciatic nerve transection¹², and urethrolisis¹³ have been described.

Vaginal distention with balloon simulating parturition resulted in increased degradative activity in the vaginal wall of nonpregnant and pregnant animals.¹⁴ Recently, stem cell therapy has emerged as a potential cure for a deficient sphincter in SUI.¹⁵ Different stem cell populations have been evaluated for this indication. The six-cell types include embryonic, muscle-derived (satellite cells), bone marrow-derived, mesenchymal, adipose, urinary, and human umbilical cord blood types.¹ Autologous adult stem cell therapy demonstrated the potential for regenerative repair of the inferior urethra for the treatment of SUI.¹⁶ ADSCs were found to differentiate into various cell types including adipogenic and osteogenic cells in the presence of lineage-specific factors.¹⁷

OBJECTIVE

This study aimed to investigate whether ADSCs are capable of nerve repair in stress urinary incontinence.

MATERIAL & METHODS

A total of 30 virgin female Wistar rats (*Rattus norvegicus*, 8-12-week-old), 150-250 grams were randomized into five groups: A (control, n= 6),

B (placebo, n= 12) and C (ADSC, n= 12). Study groups (B and C) were then divided into two subgroups after ADSCs or placebo injection based on time. First groups (B1, C1, n= 6) at two weeks and second groups (B2, C2, n= 6) at four weeks assessment, which consist of functional (VSOP) and immunohistochemical PGP 9.5 analysis.

Vaginal Distention was performed as previously described as animal SUI model.^{10,11,18-20} In brief, a lubricated urethral catheter of increasing size (10-Fr to 32-Fr) was inserted and removed to accommodate the vagina to larger capacities. A 10-Fr modified Foley catheter was then inserted into the vagina and secured with a purse-string stitch. The balloon was inflated with 3cc of water for 4 hours with a weight of 100 grams. No vaginal rupture was reported with this method.^{10,11,18-20}

Voided Stain on Paper (VSOP) as a functional assessment of SUI was measured in all animals before and after Vaginal Distention; 14 and 28 days after ADSCs or placebo injection periurethral. As previously described, The VSOP method is a useful tool for evaluating voiding behavior. First, we generated standard formula and calculated recorded stain areas using computer software Photoshop.²¹ The rats were observed for 2 hours/day (2-3 times observation period) using filter paper that placed 20 cm at the bottom of the cage. Stress or sneeze was manipulated by sprinkling chili powder around the nostrils of rat models. Urine spots on filter paper were viewed using ultraviolet light.²²

As previously described²³⁻²⁴, isolation fat tissue from the visceral fat and collected in a centrifuge tube, added 0.25% collagenase solution at 37°C for 60 minutes. The passage was performed 3-4 times to get Stromal Vascular Fraction (SVF). Combine 8 ml pellet on adipose stem cell medium (5% fetal bovine serum, 2 ng/ml basic fibroblast growth factor, 1% penicillin/streptomycin) and place it on disc plate at 37°C with 5% CO₂. Finally, several cells (adipose tissue-derived stromal vascular fraction/ADCVF) will be adhesion and growing for 2-4 days before changes of the medium).²³⁻²⁵

To facilitate the identification of the cells after their transplantation, the ADSCs were mixed with PKH2 labeling. The injection periurethral was performed one week after vaginal Distention. A low midline incision was made to expose the urethra-bladder; ADSCs were injected into both sides (5 and 7 o'clock) of the periurethral tissue around the proximal urethra. The ADSCs doses were 200.000

cells for each side in 200 ml of phosphate-buffered saline using 1 ml disposable syringe. Placebo groups were injected by 200 ml of phosphate-buffered saline in the same place. All animals were given prophylactic antibiotics (procaine penicillin 100 mg/kg intramuscular) and analgesics.²⁶

Euthanasia of all animals was performed by ketamine-xylazine overdose after functional assessment with Voided Stain on Paper (VSOP) 14 and 28 days after periurethral injection. After euthanasia, the bladder-urethra-vagina were dissected. One-half of urethra was fixed in 10% neutral buffered formalin for immunohistochemical analysis of PGP 9.5. The other was checked for PKH2 labeling using an immunofluorescence microscope.

After functional assessment using VSOP method at 14 & 28 days after injection, animals were sacrificed. The proximal urethra was removed, specimens in each group were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned into 5 µm thick slice. Paraffin sections were probed with polyclonal PGP 9.5 related antigen. All sections were deparaffinized in xylene and treated with 3% hydrogen peroxide in methanol for 10 minutes. Sections were then incubated in a humidified chamber at 4°C with the primary antibody followed by the second antibody using PGP 9.5 IHC Kit. Periurethral nerves were stained brownish. The number of periurethral nerves was counted in 3 random fields (magnification 200X) using a graticule. The average of the 3 high power fields (hpf) was calculated.

Data were reported as means ± SD. The software SPSS 17 for Windows (SPSS Inc., USA) was used for analysis. Comparisons of continuous variables among the groups were performed by one-way analysis of variance (ANOVA) for within time-point analysis. The LSD method was used to specify differences among groups. Paired t-tests were used for comparison pre-posttest functional assessment. A p-value less than 0.05 was considered to be statistically significant.

RESULTS

Voiding frequency examination was performed before and third day after VD, followed by 14 & 28 days after periurethral injection (Table 1).

Voiding behavior of group B1, B2, C1, and C2 at 14 & 28 days after injection are shown in Table 2. PostVD-PreVD differences were significant in all groups ($p < 0.05$). Voiding frequency was higher after placebo injection at Day 14 ($p = 0.002$) and Day 28 ($p = 0.64$) compared with Post VD frequency. In ADSC groups, voiding frequency was significantly lower at Day 14 ($p = 0.02$). At 28 days the frequency of ADSC group was slightly lower than postVD result, although the difference was not significant. Histological examination of the cross-section of the proximal urethra was performed on each section after immunohistochemical staining. The density of immunoreactive neurofilaments in the urethra was significantly increased at 28 days after injection of ADSCs (13.00 ± 2.37 , $p < 0.05$) compared with other groups (B1 7.33 ± 2.94 , B2 9.17 ± 2.23 , C1 6.00)

Table 1. The mean ± SD of voiding frequency.

Group	N	Pre VD	Post VD	Day 14 th	Day 28 th
PBS 14d	6	1.50 ± 0.55	3.67 ± 1.51	9.17 ± 1.84	-
PBS 28d	6	1.50 ± 0.55	3.17 ± 0.75	-	3.83 ± 2.79
ADSCs 14d	6	1.00	5.67 ± 3.08	1.83 ± 0.75	-
ADSC s28d	6	1.50 ± 0.84	6.83 ± 4.49	-	3.67 ± 2.87

Table 2. Mean difference ± SD of voiding frequency.

Pair	PBS 14d (B1)	PBS 28d (B2)	ADSCs 14d (C1)	ADSCs 28d (C2)
PostVD-PreVD	$2.17 \pm 1.83^*$	$1.67 \pm 0.82^*$	$4.67 \pm 3.08^*$	$5.33 \pm 4.97^*$
Day14 th -PreVD	$7.67 \pm 2.25^*$	-	$0.83 \pm 0.75^*$	-
Day14 th -PostVD	$5.50 \pm 2.34^*$	-	$-3.83 \pm 2.94^*$	-
Day28 th -PreVD	-	2.33 ± 3.20	-	2.16 ± 2.64
Day28 th -PostVD	-	0.67 ± 3.27	-	-3.16 ± 5.27

*: Significant, $p < 0.05$

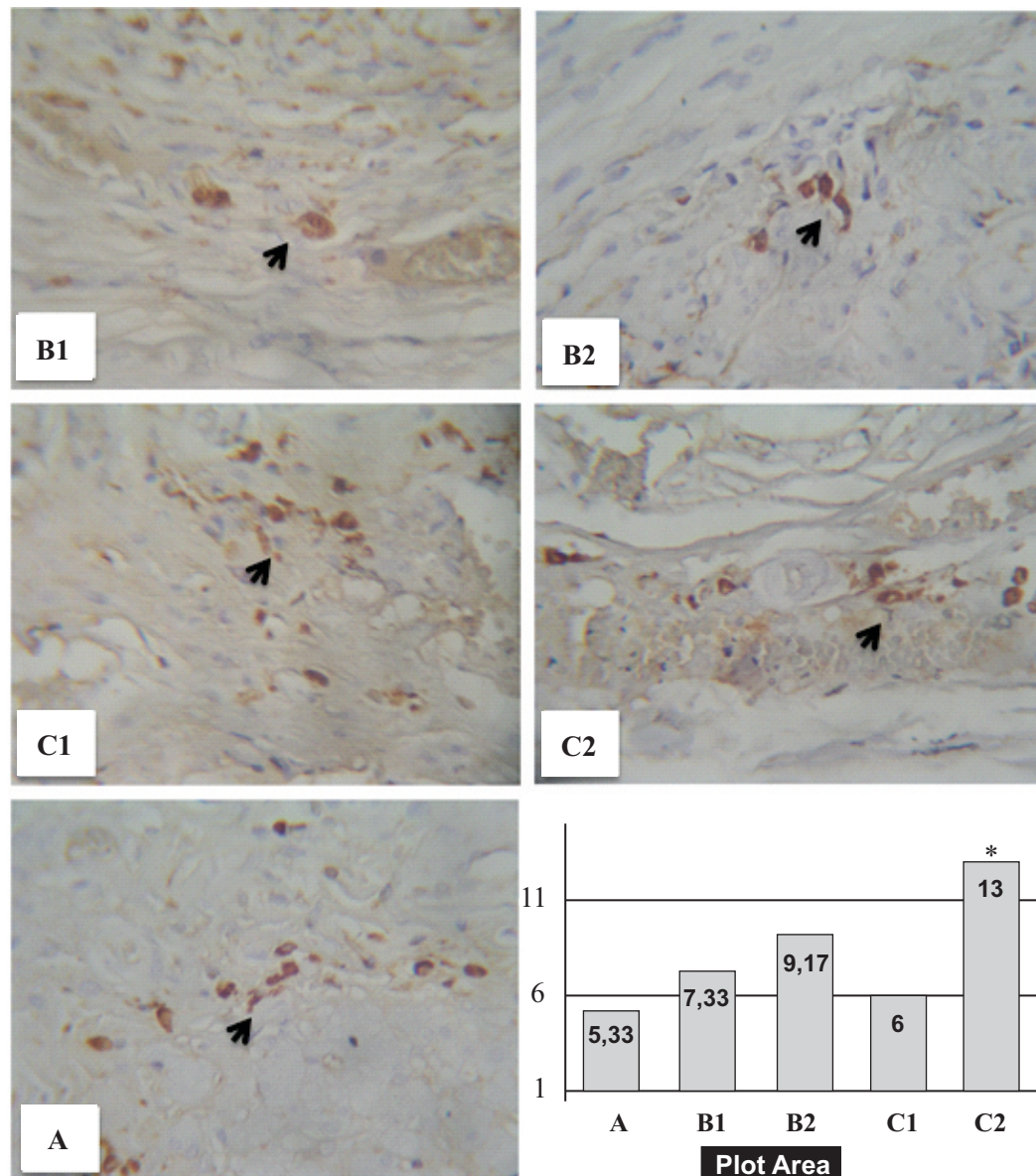


Figure 1. Histochemical analysis of the expression of PGP 9.5 related antigen and increase density of neurofilament after injection of ADSCs. Peripheral neural was stained brownish with PGP 9.5. (A: control; B1: PBS Day 14th; B2: PBS Day 28th; C1: ADSCs Day 14th; C2: ADSCs Day 28th).
* $p < 0.05$ vs PBS group (ANOVA-LSD)

DISCUSSION

In this study, we demonstrated that Vaginal Distention could induce SUI in female rats. Vaginal Distention cause similar effect with pudendal nerve crush. This animal SUI model results in decreased LPP and nerve degeneration near the External

Urethral Sphincter. The ultrastructural appearance of neurodegeneration using electron microscope were the same in both groups (VD-pudendal nerve crush).¹¹ VD has been found to cause neurodegeneration, necrosis in both smooth and striated musculature.^{10,19} The main limitation of this model is the short durability of the functional aspect,

with a recovery within 6 weeks in most cases.²⁷ Disorder of sphincter muscle seems to be the problem in Stress Urinary Incontinence. Vascularization and innervation of the sphincter complex play an important role for SUI.²⁸ Biomechanical changes observed for acute SUI may stem from functional, structural, neural, or vascular alterations that result from VD.^{7,11,19}

The VSOP method is a useful tool for evaluating voiding behavior of the mouse. We regenerated the standard formula to analyze rats voiding. Before Vaginal Distention (VD), voiding frequency was once or twice in two hours. After VD, voiding frequency was significantly increased ($p < 0.05$). This information showed us that our VD procedure was successful. After injection of ADSCs, there was significant improvement in 14 days after injection (C1) using VSOP method. VSOP method is an alternative method to measure urethral function assessment. It gives information about the mouse's voiding behavior such as interval and volume of micturition. These data showed ADSCs injection periurethral repair urethral function using VSOP method parameter.

Adipose-derived stem cells have been shown to possess self-renewing capacity, long term viability, and multipotential. Adipose tissue is derived from mesoderm and contains a supportive stroma of regenerative pluripotent progenitor cells. Adipose tissue is easily accessible and overwhelmingly abundant and can reliably differentiate into myogenic, leiomyogenic, adipogenic, osteogenic, chondrogenic, and neurogenic lineage when cultured in specific condition.^{24,29} Adipose-derived stem cells (ASCs) are a subset of mesenchymal stem cells (MSCs) that can be obtained easily from adipose tissues and possess many of the same regenerative properties as other MSC.³⁰ In Stem Cells, Muscle derived Stem Cells and Adipose-derived Stem Cells were mostly used. These cells have important features such as can be generated from autologous tissue, easily accessible, well-characterized, and support regeneration of target tissue of patients with SUI.³¹ An increasing number of studies have demonstrated that a paracrine effect rather than stem cell differentiation is the main factor resulting in their therapeutic effect.³² In our study, we demonstrated that allogenic ADSCs transplanted into periurethral could differentiate into nerve cells. ADSCs groups in our 28-day trial (C2) had a significant density of neurofilaments in periurethral.

The neurogenic hypothesis has a fundamental bearing on the etiology of SUI. This hypothesis postulates that the weakness of the sphincter mechanism is the result of chronic partial denervation of the sphincter muscles of the pelvic floor.³³ Christian et al. reported that SUI in a woman is associated with a significant reduction in total innervation of the muscle and paraurethral epithelium.³⁴

CONCLUSION

We observed that vaginal distention cause increase of voiding frequency as a sign of Stress Urinary Incontinence. Injection of Adipose-derived Stem Cells increase the density of neurofilament in periurethral and improve voiding behavior. This treatment could be used as an alternative for Stress Urinary Incontinence.

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