TRANSPLANTATION OF ALLOGENIC ADIPOSE-DERIVED STEM CELL IMPROVE URETHRAL MUSCLE-COLLAGEN RATIO AFTER VAGINAL DISTENSION

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ABSTRACT

Objective: *To investigate the effects of allogenic adipose-derived stem cell (ADSC) transplantation on urethral tissue regeneration after vaginal distension in a rat model.* ***Material & Method:*** *32 female wistar rats underwent vaginal distension (VD) for 4 h. Subsequently, PKH-labeled ADSCs (2 x 105 in 200 µl PBS, ADSC group, n = 18) or PBS (200 µl, placebo group, n = 18) were injected into periurethral tissue. Two and four weeks following transplantation (ADSC or PBS), voiding behavior (frequency) and muscle-collagen ratio of urethral tissue were measured to evaluate urethral sphincter regeneration. Data were analysed by paired-t test, one-way ANOVA and post hoc LSD.* ***Results:*** *Mean voiding frequency was significantly different in the ADSC group at two weeks ($p < 0.0001$), but not significantly different at four weeks ($p = 0.448$) when compared with the placebo group. Masson's trichrome staining revealed that the muscle-collagen ratio of urethral tissue was not significantly different between study groups at two or four weeks ($p = 0.053$ or $p = 0.166$ respectively). Muscle-urethral ratio was more specific showing a significant difference at two weeks ($p = 0.043$). There were significant differences about muscle-collagen or muscle-urethral ratio between control and placebo groups ($p < 0.05$), whereas between control and ADSC groups no significant difference was observed ($p > 0.05$).* ***Conclusion:*** *ADSC transplantation promotes urethral muscle-collagen ratio with development of striated muscle after vaginal distension, so that can improve voiding behavior in a rat model.*

Keywords: *Adipose-derived stem cell, vaginal distension, rat-SUI model, muscle-collagen ratio, voiding stain on paper.*

ABSTRAK

Tujuan: *Membuktikan pengaruh pemberian stem cell mesenkimal jaringan lemak (ASDC/Adipose-Derived Stem Cell) terhadap proses regenerasi uretra pasca distensi vagina pada rattus norvegicus.* ***Bahan & cara:*** *Sebanyak 32 ekor tikus wistar betina menjalani prosedur distensi vagina dan sisanya 8 ekor sebagai kelompok kontrol tanpa perlakuan. Kelompok perlakuan mendapatkan injeksi ADSC atau plasebo periuretra. Pemeriksaan setelah hari ke-14 dan ke-28 meliputi perhitungan frekuensi miksi dan rasio tebal otot-kolagen jaringan uretra. Analisa data dengan menggunakan uji-t berpasangan, ANOVA dan post hoc LSD.* ***Hasil:*** *Rerata frekuensi miksi kelompok ADSC berbeda bermakna pada hari ke-14 ($p < 0.0001$), namun tidak berbeda bermakna pada hari ke-28 ($p = 0.448$) bila dibandingkan dengan kelompok plasebo. Secara morfologi, rasio otot-kolagen tidak menunjukkan perbedaan yang bermakna antara kelompok ADSC dengan kelompok plasebo, baik pada hari ke-14 ($p = 0.053$) maupun hari ke-28 ($p = 0.166$), sedangkan rasio otot-uretra lebih spesifik dengan menunjukkan perbedaan yang bermakna pada hari ke-14 ($p = 0.043$). Namun apabila dibandingkan rasio otot-kolagen maupun rasio otot-uretra pada kelompok kontrol tanpa perlakuan, terdapat perbedaan yang bermakna dengan kelompok plasebo ($p < 0.05$), sedangkan dengan kelompok ADSC tidak berbeda bermakna ($p > 0.05$).* ***Simpulan:*** *Pemberian stem cell mesenkimal jaringan lemak meningkatkan pertumbuhan serat otot sirkuler (striated muscle) jaringan uretra yang sebelumnya mengalami kerusakan akibat distensi vagina, sehingga mampu memperbaiki fungsional miksi pada tikus wistar.*

Kata kunci: *Adipose-derived stem cell, distensi vagina, rat-SUI model, rasio otot-kolagen, voiding stain on paper.*

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INTRODUCTION

Stress urinary incontinence (SUI) is defined as involuntary leakage or loss of urine during activities, such as exertion, coughing, sneezing or laughing, because of increase intra-abdominal pressure without contraction of detrusor muscle, is a common condition that can impact on health, social and economic problems which will ultimately affect the quality of life.[1,2] Several epidemiological studies in Europe and the United States (1981-2000) were reviewed by Hunskaar et al revealed the overall prevalence of urinary incontinence in women ranged from 10-40%.[3] The National Health and Nutrition Examination Survey (NHANES) between 2001-2004 reported prevalence of any urinary incontinence among 4229 women over 20 years-old was 49.6%, with 49.8% suffering from SUI, 15.9% and 34.3% had urge and mixed urinary incontinence.[4]

Stress urinary incontinence in women occurs due to vesico-urethral hypermobility and/or intrinsic sphincter deficiency (ISD). Both of these mechanisms may occur during pregnancy or vaginal delivery in women with multiparity, delivery with vacuum or forceps, prolonged labor, or a large baby (> 4000 g).[2] Hansen et al investigated prevalence of any incontinence during pregnancy and found 32.1% in primiparous women and 13.8% in nulliparous women. One year after vaginal delivery, prevalence was reported up to 29.3% in primiparous and 16.6% in nulliparous women. SUI is the most frequent type of urinary incontinence (during pregnancy were 23.9% vs 7.3% and one year after delivery 15.8% vs 8.3%, respectively in primiparous and nulliparous women).[5]

Currently, the therapeutic modalities for SUI still consists of non-surgical and surgical procedures. But side effects or complications often arise after surgical procedures, despite of intrinsic sphincter deficiency pathology that is commonly found in patients with SUI still can't be corrected.[6-8] In the last decade, a medical technology of tissue engineering has developed stem cell therapy for the management of SUI. Its use was first studied by Cannon et al in 2003 with periurethral allogenic stem cell transplantation in rat model that have been manipulated to induce SUI.[9,10] Furthermore, several studies were applied to understand pathophysiology of SUI and stem cell therapy for the management of SUI that were still limited in animal models,[10-13] but fewer have been studied also in humans who suffered from SUI.[14,15]

Particularly in Indonesia, studies of stem cell therapy in the urologic disorders are very rare. With these backgrounds, we studied in female wistar rat model suffering from SUI by vaginal distension procedure, to investigate the effect of allogenic adipose-derived stem cell (ADSC) transplantation on urethral tissue regeneration, both functionally and morphologically.

OBJECTIVE

This research is aim to investigate the effects of allogenic adipose-derived stem cell (ADSC) transplantation on urethral tissue regeneration after vaginal distension in a rat model.

MATERIAL& METHOD

A total of 40 virgin female Wistar rats (rattus norvegicus, 8-12 weeks-old) weighing 150-250 grams were randomized into three groups, A (control, n = 8), B (placebo, n = 16) and C (ADSC, n = 16). Study groups (B and C) were then divided into two sub-groups after stem cell or placebo injection, i.e. first group (B1, C1, n = 8) at two-weeks and second group (B2, C2, n = 8) at four-weeks examination, which consist of voiding frequency and urethral tissue histology.

Thirty two rat models were anesthesized with a mixture of ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg). To avoid rupturing the vagina, previously it was dilated using foley catheter from 8-Fr until 24-Fr size with lubrication. Subsequently a modified 10-Fr foley catheter was inserted into the vagina, the balloon was distended with water to 3 ml and the catheter was fixed with circumferential sutures around the urethral meatus. Catheter was retained in the vagina for 4 hours with a weight of 100 grams, with expected pressure and ischemia on urethral wall.[16,17]

Voiding frequency examination carried out by the method of VSOP (voiding stain on paper), using the filter paper placed ± 20 cm at the bottom of the cage and the rats were observed for 2 hours/day (among sunset time), 2-3 times observation periods for each rat models. Stress or sneeze manipulation was performed by sprinkling chili powder around the rat nostrils. Urine spots on the filter paper were viewed using ultraviolet light (fluorescence) and voiding time and interval noted.[18,19] This procedure was performed before and third day after VD

procedure, afterwards at two-weeks and four-weeks after allogenic ADSC or placebo injection.

Fat tissue were isolated from the abdominal visceral fat, collected in a centrifuge tube and added 0.2% collagenase solution at 37ºC for 60 minutes. Passage was performed several times (3–4 times) to isolate the stromal-vascular fraction. In the last rinsing, 8 ml pellet was resuspended on adipose stem cell medium (containing 5% fetal bovine serum, 2 ng/ml basic fibroblast growth factor, 1% penicillin/streptomycin) and placed on a 100 mm x 20 mm T-25 flask plate at 37ºC with 5% CO2 (humidified incubator). Finally, several cells (adipose tissue-derived stromal vascular fraction/ADCVF) will adhere and grow for 2-4 days before change of the medium.[20]

After an midline abdominal incision, allogenic ADSCs were injected into both sides (3 and 9, or 5 and 7 o'clock) of the periurethral tissue around the proximal urethra in a group C at 1 week after VD procedure. The ADSCs dosages were 200,000 cells in 200 mL of phosphate-buffered saline using 1 ml syringe. A group B only injected 200 mL of phosphate-buffered saline periurethral in the same place. All rat models were given prophylactic antibiotics (Procaine Penicilline 100 mg/kg intramuscular) and analgesics.[13]

Two-weeks (in first sub-group) and four-weeks (in second sub-group) after injection of allogenic ADSC or placebo, 16 rats in each sub-group were sacrificed by ether before surgery. Tissue material was isolated from the proximal urethra ± 1 cm of length, fixed in 10% buffered formalin (pH 7.4), embedded in paraffin blocks and sectioned into ± 5 µm thick slices. Paraffin sections were stained with Masson's trichrome to distinguish the structure of muscle layer or rhabdo sphincter (red) and collagens (green or blue). Quantitative measurements were made using graticule and then converted to micrometers in size (µm) using a computer software.

The data were reported as means ± SD. Rat voiding frequency data were compared pre and post-test in the same group and between study groups at the same time (14-days and 28-days). While muscle-collagen and muscle-urethral ratio data were compared post-test only between group A (control), B (placebo) and C (ADSC) at the same time. The software SPSS 17.0 for windows was used for analysis with paired t-test and ANOVA, while the comparison between the study groups using a multivariate test and post hoc LSD test for a more specific 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 procedure, followed at 14-days and 28-days after administration of placebo or periurethral ADSC. The results are reported as mean ± SD (see table 1, with 8 rat models were drop-out).

Table 2-5 revealed the same results, which a mean of voiding frequency were increased in the rat models after VD procedure compared before

Table 1. The mean ± SD of voiding frequency.

Group	n	Pre VD	Post VD	14-days	28-days
Control	7	-	-	-	-
PBS 14-days	7	1.14 ± 0.378	2.29 ± 0.488	3.43 ± 0.976	-
PBS 28-days	6	1.17 ± 0.408	2.50 ± 0.548	-	2.00 ± 0.894
ADSC 14-days	6	1.00 ± 0.632	2.83 ± 0.752	1.67 ± 0.516	-
ADSC 28-days	6	0.83 ± 0.753	3.33 ± 0.817	-	1.67 ± 0.516

Table 2. Mean difference of voiding frequency in the 14-days placebo group (B1).

Pair	Mean difference	SD	t value	p
Post VD - Pre VD	1.14	0.378	8.000	< 0.0001
Day 14 - Pre VD	2.29	1.112	5.435	0.002
Day 14 - Post VD	1.14	1.069	2.828	0.030

Table 3. Mean difference of voiding frequency in the 28-days placebo group (B2).

Pair	Mean difference	SD	t value	p
Post VD - Pre VD	1.33	0.516	6.325	0.001
Day 28 - Pre VD	0.83	1.169	1.746	0.141
Day 28 - Post VD	-0.50	1.049	-1.168	0.296

Table 4. Mean difference of voiding frequency in the 14-days ADSC group (C1).

Pair	Mean difference	SD	t value	p
Post VD - Pre VD	1.83	0.753	5.966	0.002
Day 14 - Pre VD	0.67	0.516	3.162	0.025
Day 14 - Post VD	-1.17	0.983	-2.907	0.034

Table 5. Mean difference of voiding frequency in the 28-days ADSC group (C2).

Pair	Mean difference	SD	t value	p
Post VD - Pre VD	2.50	1.049	5.839	0.002
Day 28 - Pre VD	0.83	0.408	5.000	0.004
Day 28 - Post VD	-1.67	0.817	-5.000	0.004

Table 6. Mean difference of voiding frequency between study group.

The study group	Mean difference	Std. error	Value of F	p
PBS D_{14} – ADSC D_{14}	1.76	0.384	5.839	< 0.0001
PBS D_{28} – ADSC D_{28}	0.33	0.422	1.000	0.448

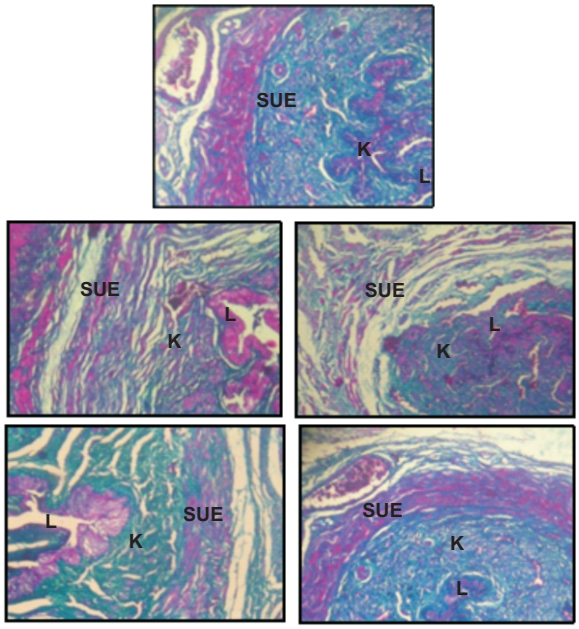

Figure 1. Cross-section of urethral tissue in rat models, Masson'strichrome staining, at 100x magnification with light microscope. Negative control group (above), placebo group at 14-days (middle-left), placebo group at 28-days (middle-right), ADSC group at 14-days (bottom-left), ADSC group at 28-days (bottom-right). SUE (external urethral sphincter), K (connective tissue collagen), L (urethral lumen).

procedure ($p < 0.05$). The voiding frequency was increased at 14th day after placebo administration (3.43 ± 0.976) compared with before (1.14 ± 0.378, $p = 0.002$) or after VD procedure (2.29 ± 0.488, $p = 0.030$), whereas at 28th day after placebo administration showed no significant difference (tables 2 and 3). In the ADSC group, a mean of voiding frequency were reduced significantly at 14th day (1.67 ± 0.516) and 28th day (1.67 ± 0.516) after stem cell administration compared with voiding frequency after VD procedure (2.83 ± 0.752 and 3.33 ± 0.817 respectively, $p < 0.05$) (table 4 and 5).

If the differences of voiding frequency in rat models were compared between study groups, significant differences seen in the group between the 14th day after placebo administration compared with ADSC group (3.43 ± 0.976 vs 1.67 ± 0.516, $p < 0.0001$), but at 28th day there is no significant difference (2.00 ± 0.894 vs 1.67 ± 0.516, placebo vs ADSC, $p = 0.448$) (table 6).

The muscle-collagen ratio is obtained by divided the measure of muscle and collagen thickness. Muscle-collagen ratio was highest in the untreated control group (0.75 ± 0.139), subsequently in the 28-days ADSC group (C2), 14-days ADSC group (C1), 28-days placebo group (B2) and lowest ratio in the 14-days placebo group (B1), respectively $0,69 \pm 0,209$, 0.67 ± 0.111, 0.56 ± 0.183, and 0.50 ± 0.135 (Fig. 2). The muscle-collagen ratio was significantly different only between the untreated control group compared with the placebo group, both of at 14-days and 28-days ($p < 0.05$).

The muscle-urethral ratio is obtained by divided the measure of muscle and the urethra wall thickness. Muscle-urethral ratio was highest in the untreated control group (0.43 ± 0.046), and then followed in 28-days ADSC group (0.40 ± 0.070), 14-days ADSC group (0.39 ± 0.042), 28-days placebo group (0.35 ± 0.073) and lowest ratio in the 14-days placebo group (0.33 ± 0.060) (Fig. 3).

The muscle-urethral ratio was significantly difference between the untreated control group compared with the placebo group, both at 14-days and 28-days ($p < 0.05$), and between the 14-days placebo group compared with ADSC group ($p < 0.05$).

DISCUSSION

Stress urinary incontinence is very common in women during pregnancy and after vaginal delivery, especially related to large baby and

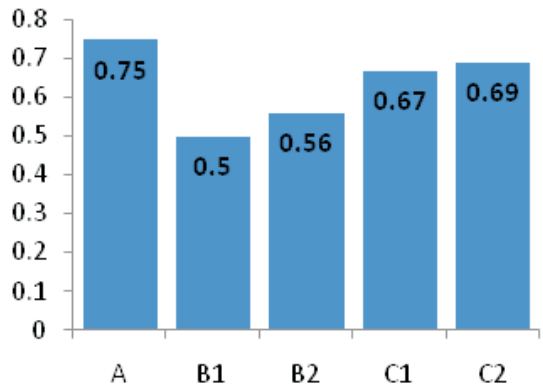

Figure 2. Muscle-collagen ratio chart.

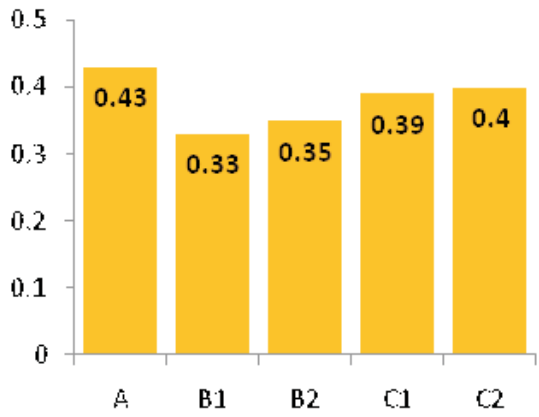

Figure 3. Muscle-urethral ratio chart.

prolonged duration of the second stage of labor, caused by damage to the external urethral sphincter, the skeletal muscle as well as the smooth muscle of the urethra (intrinsic compartment), vaginal smooth muscle and other pelvic floor structures via direct compression or indirect hypoxia and ischemic mechanisms. Furthermore, this pathogenesis can be studied in animal models with vaginal distension procedure such as performed in this study. Several other studies about this method has proven effects of hypoxia and ischemia of the urethral tissue due to compression by balloon dilatation in the vagina (pressure-induced ischemia), causing damage of blood vessels or venous plexus in the submucosal layer, atrophy of the smooth muscle fibers and urethral striated muscle or rhabdo-sphincter in the muscular is layer and damage of the nerve cells of periurethral ganglia plexus. Ensuing weakness of external urethral sphincter and decrease of urethral

resistance, and leads stress urinary incontinence in the animal model (animal-SUI model).[17,21]

The results in this study showed that vaginal distension procedure can cause of symptoms similar with SUI in rat models. There is a significantly increased voiding frequency after VD in each study group compared to before VD. A significant increase of voiding frequency was also occurs at the 14th day after placebo administration, although at 28th days there wasn't significantly different. While in the ADSC group, transplantation of mesenchymal stem cells can be reduced of voiding frequency significantly at two-weeks and four-weeks when compared with the voiding frequency after VD procedure (or before the injection of stem cells).

The results of histologic examination showed that both of muscle-collagen ratio and muscle-urethral ratio in the placebo group were significantly lower when compared with the untreated control group, whereas in the ADSC group there was no significant difference compared with the control group. These results demonstrate that transplantation of stem cells capable to repairing and regenerating of muscle fibers in the muscularis layer of the urethra wall which previously damage due to vaginal distension. It is reinforced by the results of voiding frequency in the ADSC group were reduced significantly at the two-weeks until the four-weeks. Contrast, improvement was not observed in the placebo group, and reinforced by the results of voiding frequency after the second week of placebo administration was still increased significantly. ADSCs are multipotent cells which possess numerous advantages in myoblast inducement and autologous cell implantation. Adipose tissues are abundant, can easily be accepted by patients, and are obtained through a simple adipectomy. In addition, ADSCs are so abundant in the adipose tissues that only a minimal amount of adipose tissue is needed. ADSCs are also highly proliferative. Primary ADSCs can attach after 12 hours of isolation and quickly enter the log growth phase. ADSCs are homogenous and have uniform morphology, which help to prevent unpredictable side effects after implantation. Also, because they are highly proliferative and homogenous, ADSCs can easily be differentiated after induction. Finally, the quality of ADSCs is only minimally affected by age. Adipose tissues with abundant cells can readily be obtained, and can easily regenerate for multiple uses. ADSCs may be, therefore, more advantageously suitable in treating stress incontinence through the process of homing and differentiation myoblasts.[11]

Vaginal distension procedure in this study apparently causes acute and reversible urethral damage, according to the time and phases of wound healing (inflamation, proliferation and remodeling). There was spontaneous healing at fourth week after VD procedure and placebo administration, voiding frequency was reduced at four-weeks in placebo group and muscle-collagen ratio were not significantly different between placebo and ADSC group, both of two and four-weeks. The other studies revealed similar results regarding the reversibility of vaginal distention effects due to compression for more than 1 hour (reperfusion injury), showing wide spread damage or disruption of striated muscle and thinning of the muscle fibers beginning on the fourth day,[16,21,22] but gradually returned to normal morphology after fourth week, sixth week or over three months.[11,16,23]

CONCLUSION

Transplantation of allogenic adipose-derivedstem cells (ADSC) promotes higher urethral muscle-collagen ratio with development of striated muscle after vaginal distension, which can improve the voiding behavior in a rat model.

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