EFFECT OF ADIPOSE-DERIVED STEM CELL TRANSPLANTATION ON URETHRAL MICROVESSELS AND VOIDING FUNCTION

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ABSTRACT

Objectives: *To examine the effects of adipose-derived stem cell (ADSC) transplantation on urethral tissue microvessels and voiding function on artificial stress urinary incontinence (SUI) in rats.* ***Material & methods:*** *Twenty-five of 32 female wistar rats underwent vaginal distension as animal-SUI model. ADSCs were isolated from the periovarian fat, examined for stem cell properties, and labeled with PKH-2. Seven rats without vaginal distension and periurethral ADSC injection were controls. Twelve rats received periurethral injection of phosphate buffer saline as placebo and 13 rats received periurethral injection of ADSCs. Fourteen and 28 days later, voided volume was measured with voided stain on paper and microvessel density was measured with immunohistochemical analysis (factor VIII R-Ag).* ***Results:*** *Voided volume analysis showed that rats in the placebo group had abnormal voided volume compared to normal rats in day 14 (168.5 ± 119.05µl vs 621.21 ± 283.85µl; $p < 0.05$), and insignificant improvement of voided volume compared to normal rats in day 28 (852.09 ± 626.7µl vs 868.49 ± 578.0µl; $p > 0.05$). While the ADSC group only showed significant improvement of voided volume compare to abnormal rats in day 14 (379.35 ± 191.74µl vs 228.18 ± 56.26µl; $p < 0.05$). Immunohistochemical analysis showed that microvessel density was higher in the ADSCs group compared to placebo group in days 28 (12.86 ± 2.5 vs 9.50 ± 1.64; $p < 0.05$).* ***Conclusion:*** *ADSC transplantation promotes improvement in voided volume and urethral microvessel in the rat-SUI model.*

Keywords: *Adipose-derived stem cell, vaginal distension, voided stained on paper, microvessel density.*

ABSTRAK

Tujuan: *Mengevaluasi pengaruh transplantasi adipose-derived stem cell (ADSC) pada microvessel jaringan uretra dan fungsi voiding pada stress urinary incontinence (SUI) buatan pada tikus.* ***Bahan & cara:*** *Dua puluh lima dari 32 tikus wistar betina menjalani distensi vagina sebagai model SUI binatang. ADSC diisolasi dari lemak peri-ovarium, dievaluasi untuk bahan sel punca, dan diberi label PKH-2. Tujuh tikus tanpa distensi vagina dan injeksi ADSC periuretral diobservasi sebagai kontrol. Dua belas tikus menerima injeksi periuretra phosphate buffer saline sebagai plasebo dan 13 tikus menerima injeksi periuretral ADSC. Empat belas dan 28 hari kemudian, voided volume diukur dengan voided stainon paper dan microvessel density diukur dengan analisa imunohistokimia (faktor VIII R-Ag).* ***Hasil:*** *Analisa voided volume menunjukkan bahwa tikus pada kelompok plasebo memiliki voided volume abnormal dibandingkan dengan tikus normal pada hari ke 14 (168.5 ± 119.05µl vs 621.21 ± 283.85µl; $p < 0.05$), dan peningkatan voided volume yang tidak signifikan dibandingkan dengan tikus normal pada hari ke 28 (852.09 ± 626.7µl vs 868.49 ± 578.0µl; $p > 0.05$). Sementara pada kelompok ADSC hanya menunjukkan peningkatan voided volume yang signifikan dibandingkan dengan tikus abnormal pada hari ke 14 (379.35 ± 191.74µl vs 228.18 ± 56.26µl; $p < 0.05$). Analisa imunohistokimia menunjukkan bahwa microvessel density lebih tinggi pada kelompok ADSC dibandingkan dengan kelompok plasebo pada hari ke 28 (12.86 ± 2.5 vs 9.50 ± 1.64; $p < 0.05$).* ***Simpulan:*** *Transplantasi ADSC mendukung peningkatan voided volume dan urethral microvessel pada model tikus-SUI.*

Kata kunci: *Sel punca adipose-derived, distensi vagina, voided stained on paper, microvessel density.*

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INTRODUCTION

Stress urinary incontinence (SUI) is defined by ICS as involuntary leakage or loss of urine during activities, such as exertion, coughing, sneezing or laughing, because of increased intra-abdominal pressure without contraction of detrusor muscle. These condition can impact the patient health, social, economic, and furthermore will affect the quality of life.[1,2] Several studies shows that SUI event may vary among authors, according to Hunskaar et al, 77% women suffering from SUI in Norway,[3] But Dooley et al study show 49.8% suffering from SUI in America.[4]

Currently, the modalities for SUI consists of non-surgical and surgical procedures, and still surgical procedures as a gold standard. But not only side effects or complications often arise after surgical procedures, but also the results are often not permanent.[5-6]

Stem cell therapy for the management of SUI is a new alternative therapy that is still in progress. Several studies using stem cell therapy for the management of SUI in animal models,[7-9] and humans who suffered from SUI,[10,11] showed good results. In the present study, we used rats as animal SUI model to investigate the effect of adipose-derived stem cell (ADSCs) transplantation on urethral tissue regeneration, both in functional (voided volume) and morphological (microvessel) outcomes.

OBJECTIVE

To examine the effects of adipose-derived stem cell (ADSC) transplantation on urethral tissue microvessel and voiding function on artificial stress urinary incontinence (SUI) in rats.

MATERIAL & METHODS

Thirty-two virgin female wistar rats (Rattus norvegicus), age 8-12 weeks old, and weight 150-250 gr included in this study. Twenty five of 32 female wistar rats underwent vaginal distension (VD) as animal-SUI model. The rats were randomly divided into five groups: 7 rats without VD and without periurethral ADSC injection as control control/sham group (S), 12 rats received periurethral injection of phosphate buffer saline (PBS) as placebo and 13 rats received periurethral injection of ADSCs. These two groups were subdivided into two different observation times, 14 and 28 days. PBS group was subdivided into PBS_{14} (6 rats) and PBS_{28} (6 rats), and ADSC group subdivided into $ADSC_{14}$ (6 rats) and $ADSC_{28}$ (7 rats).

All rats were evaluated for voiding function (voided volume) and immunohistochemical analysis (microvessel density) at days 14 and 28. Evaluation for voiding function used “the randomized pre-test and post-test group design” while immuno-histochemical evaluation used ”the randomized post-test only control group design”.

Female wistar rats were anaesthetised with mixture of ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg) intramuscular injection. The vagina was predilated first with catheter size 10 until 24 Fr, and then a modified 10 Fr catheter was inserted into the vagina and fixed with non-absorable 4-0 suture, followed by injection of 3 ml of normal saline into the balloon catheter to compress the vaginal wall. Catheter was then leveled and attached with 100 mg of weights for 4 hours before removal.[12,13]

This method began with generation of the standard formula and analysis of rats voiding. Normal saline ranging from 20 to 560 µl was poured on a filter paper by a micropipette from a point 20 cm above the paper. The recorded stain areas were copied to another paper and scanned to computer software Adobe Photoshop for measuring the stained area on the paper as pixel area. The correlation between amount of saline and pixel area was calculated to generate a standard formula for further rats studied (figure 1).

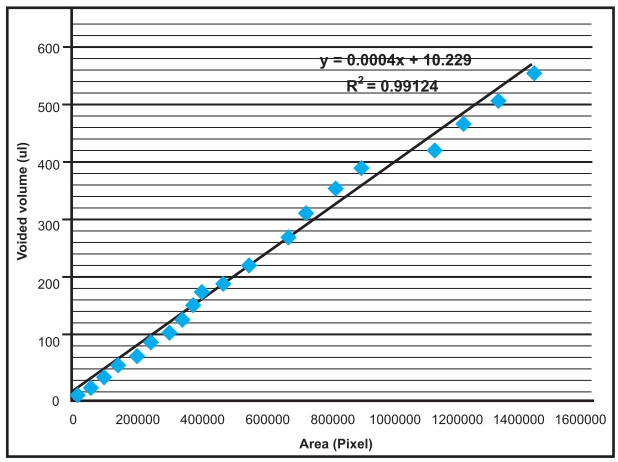

Figure 1. Linear correlation between liquid volume (l) and stained area (pixel) on the filter paper within the range of 20-560 µl (y = 2486x-20506; x: pixel area, y: voided volume).

Voiding behavior of rats was analyzed by placing the animal on a wire-netted cage cover 20 cm above the filter paper. The diameter of the wire was 1 mm, the size of nett was 1 cm, and the cage size was 30 x 40 x 30 cm. The voided time and area were recorded over 2 hours, and chilli powder was poured on rat nostrils to stimulate sneezing. Using the ultraviolet light, the recorded stain areas were copied to another paper and converted to urine volume by the formula generated as described above. The voided volume of an animal was calculated as mean value of multiple voided volume with number of voids within the examination period.[14,15]

The urethra was harvested at the indicated time period (day 14 and 28), and then tissue material was isolated from the proximal urethra ± 1 cm of length, fixed in 10% buffered formalin (pH 7.4), embedded in paraffin and sectioned (5 μm). After deparaffinized, the sections were probed with monoclonal Mouse Anti Human Von Willebrand Factor with cross reactivity with rat (FVIII-R Ag, AbD Serotec,USA). The sections were stained with 3.3-diaminobenzidine tetrachloride solution (DAB). The endothelial cells in blood vessels that stained brownish red were FVIII-R Ag (+) cells.

The number of microvessels was counted in 3 random fields at 100x magnification. The average of 3 high powered fields (hpf) was calculated and the microvessel density was defined as microvessel/hpf.[16]

ADSCs were isolated from peri-ovary and visceral fat of three 8-week-old wistar rats, and then 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 get the stromal-vascular fraction. In the last rinsing, resuspended 8 ml pellet 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 x 20mm T-25 flaskplate at 37ºC with 5% CO^2 (humidified incubator). Finally, several cells (adipose tissue-derived stromal vascular fraction/ADCVF) will be adhesing and growing for 2-4 days before change of the medium. After culturing the cells to facilitate identification of cells after transplantation, ADSCs were labeled with PKH-2. PKH-2 are fluorescent cell linker kit selectively labeling stem cells.[17] The injections were performed 1 week after vaginal distension, with a low midline abdominal incision made to expose the urethra and ADSCs (200.000 cells) or PBS were injected into both side of the periurethral tissue using a 1 ml disposible syringe at 3 and 9 or 5 and 7 o'clock.[9]

Data were analyzed using SPSS 16.0 for Windows. All voided volume and microvessel density data are presented as means ± SD. Paired-t test was used to compare the voided volume data, and One-way ANOVA was used to compare the microvessel density data, with p value < 0.05 considered as statistically significant.

RESULTS

Voided volume examination was performed before and after VD procedure, followed at 14-days and 28-days after administration of placebo or periurethral ADSCs. The results are reported as mean ± SD (table 1).

Tabel 1. Mean voided volume (μl).

Group	Pre VD	Post VD	Day 14	Day 28
PBS H_{14}	621.21 ± 283.85	246.65 ± 175.44	168.50 ± 119.05	-
PBS H_{28}	868.49 ± 578.00	287.18 ± 191.56	-	852.09 ± 626.70
ADSC H_{14}	431.05 ± 131.41	228.18 ± 56.26	379.35 ± 191.74	-
ADSC H_{28}	624.52 ± 312.09	272.88 ± 295.57	-	308.02 ± 217.18

Tabel 2. Mean difference of voided volume 14 days after PBS injection.

Pair	Mean difference	SD	t value	p value
Post VD – Pre VD	-374.56	313.47	-2.927	0.033
Day 14 – Pre VD	-452.71	272.24	-4.073	0.010
Day 14 – Post VD	-78.15	209.78	-0.913	0.403

Tabel 3. Mean difference of voided volume 28 days after PBS injection.

Pair	Mean difference	SD	t value	p value
Post VD – Pre VD	-581.31	494.54	-2.879	0.035
Day 28 – Pre VD	-16.40	263.63	-0.152	0.885
Day 28 – Post VD	564.91	548.28	2.524	0.053

Tabel 4. Mean difference of voided volume 14 days after ADSCs injection.

Pair	Mean difference	SD	t value	p value
Post VD – Pre VD	-202.87	157.75	-2.572	0.082
Day 14 – Pre VD	-51.70	292.93	-0.353	0.747
Day 14 – Post VD	221.69	175.44	3.095	0.027

Tabel 5. Mean difference of voided volume 28 days after ADSCs injection.

Pair	Mean difference	SD	t value	p value
Post VD – Pre VD	-351.64	462.70	-1.862	0.122
Day 28 – Pre VD	-316.50	362.67	-2.138	0.086
Day 28 – Post VD	16.64	360.29	0.122	0.907

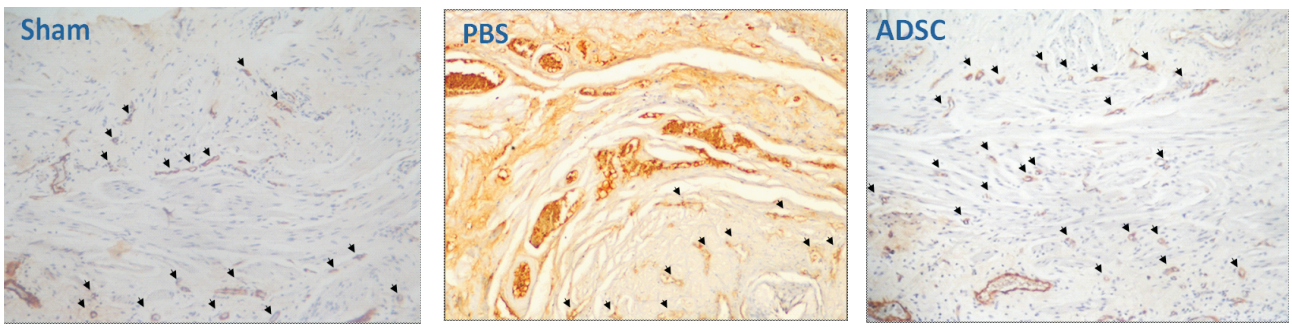

Figure 2. Cross-section of urethral tissue in rat models with immunohistochemical staining (F VIII R-Ag), at 100x magnification with light microscope. Microvessel (black arrows) in ADSC group showed significant increase ($p < 0.05$) compare to PBS (placebo) group.

In table 2 and 3 voided volume analysis showed that rats in the placebo group had abnormal voided volume compared with normal rats (pre VD) in day 14 (168.5 ± 119.05µl vs 621.21 ± 283.85µl; $p < 0.05$), and insignificant improvement of voided volume compared with normal rats in day 28 (852.09 ± 626.7µl vs 868.49 ± 578.0µl; $p > 0.05$).

While the ADSC group (tabel 4 and 5)showed significant improvement of voided volume compared to abnormal rats on day 14 (379.35 ± 191.74µl vs 228.18 ± 56.26µl; $p < 0.05$). But on day 28 in ADSC group showed no significant improvement of voided volume compared with abnormal rats (308.02 ± 217.18µl vs 272.88 ± 295.57µl, $p > 0.05$).

Cross-section of urethral tissue in rat models with immunohistochemical staining (F VIII R-Ag), showed no significant improvement of microvessel density in placebo group both in day 14 or 28, compared with sham group (9.17 ± 1.72 and 9.50 ± 1.64 vs 12.00 ± 0.82). But microvessel density was significantly higher in the ADSCs group compare to placebo group in days 28 (12.86 ± 2.5 vs 9.50 ± 1.64; $p < 0.05$).

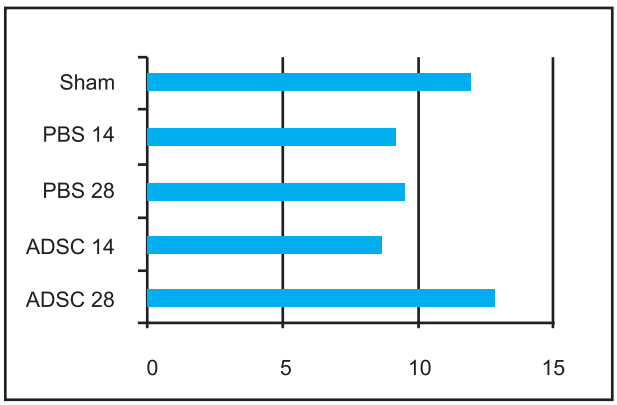

Figure 3. Microvessel density of each group compared to sham group.

DISCUSSION

The impairment of voiding function and vascular damage in SUI occurs due to trauma to the connective tissue support due to the mechanical process of vaginal childbirth.[2,18] The effect of vaginal distension for 3 hours in the rats showed damage to periurethral tissue (muscle, vascular, nerve) after the third day, the seventh day until the fourteenth day post vaginal distention, but returned to normal spontaneously after twenty-eighth day.[19] This is caused by the presence of pressure-induced hypoxia and associated ischemia, necrosis or reperfusion injury to the urethra tissue after vaginal distension procedure, causing biomechanical changes that include changes instructure, nerves, vessels and functional urethra.[20,21]

Several studies have suggested duration required for periurethral tissue healing and repair of the micturition pattern in animal SUI models. Obinata et al showed in his research that the improvement of micturition pattern (measured by examination of abdominal leak point pressure/ ALPP) will decrease from day 7 today 14 after vaginal distension in the rat-SUI models, but increased again after day 28 without the provision of stem cells. In the group given stem cells, there is an increased ALPP on day 14, it is shown that a significant improvement of micturition function associated with stem cell administration in animal SUI models after day [14.19]

Study from Sugino et al, in mice as urinary frequency model created with intraperitoneal administration cyclophospamide, found a significant decrease in micturition volume when compared with controls (127.8 ± 100.0 and 362.7 ± 51.9 μl; $p < 0.001$) and found a significant decrease in micturition interval (10.30 ± 3.10 and 4.47 ± 1.70 min). The conclusion of this study, found not only increased micturition frequency, but also decreased micturition volume.[14] This is analogous to the condition of SUI in which pre-existing conditions affects the continence mechanism.[2]

Although in this present study, the method of measuring changes in micturition patterns not using standard urodynamics, but VSOP sufficiently demonstrated changing patterns of micturition. Linetal, defines animal models of SUI as continuous/intermittent urine leakage with low intravesical pressure, that cause decreased micturition volume and increased frequency of micturition.[8]

In this present study, microvessel density improvement at day 28 was not followed by improvement of voided volume at day 28, because of improvement of the voided volume takes a longer time. This is shown in research by Fu Q et al, voided volume has not returned to normal in the placebo group at 12 weeks, while improvement was seen in stem cell administration group at week 12 compared to control group.[7] Longer observation is required to evaluate improvement of voided volume.

The purpose of ADSC therapy is to differentiate according to the track, like a cell or tissues such as osteoblasts, chondroblasts, myoblasts, or adipose cells,[7,19] but some literature mentions that ADSCs has the ability of angiogenesis.[22,23] The ability of ADSCs to differentiate outside the track is called transdifferentiation[7] or plasticity.[23]

ADSCs role in the improvement of microvessel density in addition to the nature of the angiogenesis, also due to growth factors. Growth factors that play a role in the process of angiogenesis is VEGF (vascular endothelial growth factor). VEGF is a growth factor that is pro-angiogenic. Angiogenesis can be triggered due to ischemia of tissues, tissue damage, or malignancy. In this study, increased expression of VEGF in tissue trauma or ischemia, triggers activation of the glands in the surrounding tissue (paracrine), there by stimulating the growth of new microvessels.[22,24]

According to Goldman et al, activation of these paracrine factors caused emergence of several effects such as immunomodulatory, anti-apoptosis, angiogenesis, antifibrosis, chemo attractive, and stimulating proteins that leads to new cell growth.[24] Thus, significant improvement of microvessel

density in the group administered ADSCs at observation day 28, suggests that stem cell therapy can regenerate the periurethral tissue, in this case the number of microvessels.[7-9]

Ning et al. observed that transplantation of ADSCs into corpus cavernosum of male rats, help differentiation of endothelial cells that are found in the sinusoidal endothelium. Therefore, increasing the amount of endothelial cells stimulates new vascularization.[25] Additional in vivo studies in rats as animal-models of SUI using mesenchymal stem cells, obtained a significant increase of microvessel density on day 28 ($p < 0.01$) between the control group compared with the group given fibrin glue (FG) + muscle-derived stem cell (MDSC) or the group given only MDSC.[16]

CONCLUSION

ADSC transplantation promotes improvement of voided volume and urethral microvessel in the rat-SUI model, but there are some lacking data about how long the ADSCs will take effect on restoring voiding function.

REFERENCES

1. Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, et al. The standardisation of terminology of lower urinary tract function: Report from the standardisation sub-committee of the international continence society. Neurourology and Urodynamics. 2002; 21: 167-78.
2. Chapple CR. Milsom I. Urinary incontinence and pelvic prolapse: Epidemiology and pathophsiology. In: Wein AJ, Kavoussi LR, Novick AC, Partin AW, Peters CA (ed). Campbell-Walsh Urology, 10th ed. Philadelphia: Elsevier-Saunders; 2012. p. 1871-95.
3. Hunskaar S, Burgio K, Diokno A, Herzog AR, Hjalmås K, Lapitan MC. Epidemiology and natural history of urinary incontinence in women. Urology. 2003; 62 (Suppl 4A): 16-23.
4. Dooley Y, Kenton K, Cao G, Luke A, Durazo-Arvizu R, Kramer H, et al. Urinary incontinence. Prevalence: Results From the National Health and Nutrition Examination Survey. J Urol. 2007; 179: 656-61.
5. Chapple CR, Bhargava S, Andersson KE. Medical, behavioral and minimally invasive therapy- a Urologist's view. In: Becker HD, Stenzl A, Wallwiener D, Zittel TT (ed). Urinary and Fecal Incontinence, An Interdisciplinary Approach. Berlin Heidelberg: Springer-Verlag; 2005. p. 182-96.
6. Payne CK. Conservative management of urinary incontinence: Behavioral and pelvic floor therapy, urethral and pelvic devices. In: Wein AJ, Kavoussi LR, Novick AC, Partin AW, Peters CA (Ed). Campbell-Walsh Urology, 10th ed. Philadelphia: Elsevier-Saunders; 2012. p. 2004-25.
7. Fu Q, Song XF, Liao GL, Deng CL, Cui L. Myoblasts differentiated from adipose-derived stem cells to treat stress urinary incontinence. J Urol. 2009; 75: 718-23.
8. Lin G, Wang G, Banie L. Treatment of stress urinary incontinence with adipose tissue derived stem cells. Cytotherapy. 2010; 1: 88-95.
9. Zhao W, Zhang C, Jin C, Zhang C, Kong D, Xu W. Periurethral injection of autologous adipose-derived stem cells with controlled-release nerve growth factor for the treatment of stress urinary incontinence in a rat model. EuroUrol. 2011; 59: 155-63.
10. Mitterberger M, Pinggera GM, Marksteiner R, Margreiter E, Fussenegger M, Frauscher F, et al. Adult stem cell therapy of female stress urinary incontinence. Euro Urol. 2008; 53: 169-75.
11. Yamamoto T, Gotoh M, Hattori R, Toriyama K, Kamei Y, Iwaguro H, et al. Periurethral injection of autologous adipose-derived stem cells for the treatment of stress urinary incontinence in patients undergoing radical prostatectomy: Report of two initial cases. Int. J Urol. 2010; 17: 75-82.
12. Lin AS, Carrier S, Morgan DM, Lue TF. Effect of simulated birth trauma on the urinary continence mechanism in the rat. Urology. 1998; 52: 143-51.
13. Cannon TW, Wojcik E, Ferguson, C, Saraga S, Thomas C, Damaser M. Effect of vaginal distention on urethral anatomy and function. BJU Int. 2002; 90: 403-7.
14. Sugino Y, Kanematsu A, Hayashi, Haga H, Yoshimura N, Yoshimura K. Voided stain on paper method for analysis of mouse urination. Neurourol. Urodynam. 2008; 7: 548-52.
15. Heidkamp MC, Leong FC, Brubaker L, Russell B. Pudendal denervation affects the structure and function of the striated urethral sphincter in female rats. Int Urogynecol J. 1998; 9: 88-93.
16. Xu Y, Song YF, Lin ZX. Transplantation of muscle-derived stem cells plus biodegradable fibrin glue restores the urethral sphincter in a pudendal nerve-transected rat model. Braz. J. Med. Biol. Res. 2010; 43: 1076-83.
17. Yamamoto N, Akamatsu H, Hasegawa S, Yamada T, Nakata S, Ohkuma M, et al. Isolation of multipotent stem cells from mouse adipose tissue. Journal of Dermatological Science. 2007; 48: 43-52.
18. Phillips C, Monga A. Childbirth and the pelvic floor: The gynaecological consequences. Reviews in Gynaecological Practice. 2005; 5: 15-22.
19. Obinata D, Matsumoto T, Ikado Y. Transplantation of mature adipocyte-derived dedifferentiated fat (DFAT) cells improve urethral sphincter contractility in a rat model. Int. J. Urol. 2011; 18: 827-34.
20. Damaser MS, Whitbeck C, Chichester P, Levin RM. Effect of vaginal distension on blood flow and