Concomitant Transurethral and Transvaginal-Periurethral Injection of Autologous Adipose Derived Stem Cells for Treatment of Female Stress Urinary Incontinence: A Phase One Clinical Trial

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Abstract- Stress urinary incontinence is a common medical problem among women. The urethral closure complex and/or the supportive mechanisms are responsible for incontinence in the majority of patients. Several surgical procedures with different degrees of invasiveness and outcomes have been reported to treat the problem. Although most of these procedures are reasonably effective, a general trend towards the study of natural and biocompatible tissues is emerging over popular synthetic materials. Here we report our experience of autologous adipose-derived stem cells transplantation into the periurethral region as a new method of stress urinary incontinence treatment. Ten women with symptoms of stress urinary incontinence were treated by injections of autologous adipose-derived stem cells into the periurethral region via transurethral and transvaginal approach under urethroscopic observation. This report presents the short-term outcome of the patients. The outcome measured by pad test results, ICIQ-SF scores, and Qmax. The mean age of the participants was 45.8±8.7 years. Urinary incontinence significantly decreased through the first two, 6 and 24 weeks after the injection therapy. The difference was significant in pad test results (P<0.001) and ICIQ-SF scores (P<0.001), especially comparing results between 2 and 6 weeks and among 6 and 24 weeks, but not for 2 and 6 weeks compared to each other. Surprisingly, Qmax showed improvement after the study period (means 32.6 *vs.* 35.7; P=0.002). This study showed that injection of the autologous adipose-derived stem cells to the periurethral region is a safe, yet short-term effective treatment option for stress urinary incontinence. Further studies with longer follow up are needed to confirm its long term efficacy.

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Introduction

Urinary incontinence is a common medical problem, affecting more than 200 million patients in the world (1,2). It is much more common in female than in male population (3). International Consultation on incontinence reported that approximately 49% of all urinary incontinent women suffered from stress urinary incontinence (SUI), 22% from urge incontinence and 29% from mixed incontinence (1,4). This means that in the majority of incontinent women, the urethral closure complex, the urethra, and the rhabdosphincter should be the main target of treatment in many patients. This is especially true for those who have SUI due to intrinsic sphincter deficiency (ISD) alone, or patients with concomitant urethral hypermobility (5). The idea of injection therapy for urinary incontinence developed more than a century ago (4). Several therapies have been investigated including paraffin, polytetrafluoroethylene (Teflon), collagen and autologous fat tissue,

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chondrocytes, and muscle (6,7). An injectable agent should have special characteristics such as being nonantigenic, noncarcinogenic, not easily dissociate or migrate, and not severely inflammatory to be effective and safe to use (8,9). Choosing the most natural and biocompatible material has another importance, not to interfere with later surgical procedures. However, no biologic or synthetic material has been perfect to meet all the criteria yet (6-9). Complications and poor long-term efficacy of periurethral injections of bulking agents, lack of success in pharmaceutical treatment, complications of invasive procedures such as sling surgery lead to alternative treatments, especially those which are able to restore natural continence physiology (10-13). So, there is an increasing need for effective and minimally invasive approaches with low morbidity for the treatment of SUI. To date, there have been some well-known injection techniques: periurethral and transurethral. Although result has been promising and comparable, some parts like periurethral techniques and transurethral without cystoscope are troublesome. So, new needle localization approaches for injection has always been under investigation (14).

Stem cell based treatment has provided a new hope to overcome the limitation of current treatments. Cell therapy for the regeneration of injured tissues has recently been extensively investigated at the experimental level and a wide variety of clinical application. Stem cells are pluri or multipotent cells that are characterized by multilineage differentiation potential and self-renewal properties. Mesenchymal stem cells (MSC) are multipotent stromal cells that can differentiate into a variety of tissues, using the standard in vitro tissue culture-differentiating conditions (15-19). Additionally, MSCs express some specific surface markers as CD105, CD73, and CD90. On the other hand, there is a lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II as measured by flow cytometry (20). Cultured adipose-derived stem cells (ASCs) are a type of mesenchymal stem cells that secrete a variety of angiogenesis-related cytokines, such as vascular endothelial growth factor and hepatocyte growth factor (21). Thus far, MSC have primarily been harvested from the bone marrow, a tissue source that has many limitations including donor-site morbidity in the bone marrow, which limits the amount of marrow that can be obtained (22,23), while adipose tissue contains multipotent cells similar to MSCs (24,25) with a higher number of stem cells compared with bone marrow. Recent experimental studies indicate the role of periurethral injection of low serum cultured ASCs to increase urethral resistance (26). Yamamoto *et al*., showed the safety of periurethral injection of the autologous adipose adult stem cell as a treatment modality for male SUI in three cases (27). Mitterberger *et al*., used autologous stem cells to treat SUI which supporting data represent promising new and minimally invasive treatment modality (28). We report our experience of transurethral plus transvaginal injection of autologous ASCs under urethroscopic observation in ten women suffering from SUI, as a new, stem cell based injection therapy with simpler and more applicable technique.

Materials and Methods

After institutional review board (IRB) approval, written informed consent was provided by all patients. A total of ten women complaining of long term SUI, resistant to conservative and medical therapy who admitted at the urology clinic of Sina hospital, enrolled in the study. This study is a phase one clinical trial; the registration number is 14154 that registered in Iranian Registry of Clinical Trials. Inclusion criteria were age between 30 to 60 years, history of SUI (more than 2 years), being unresponsive to conservative measures (like behavioral therapy, pelvic floor rehabilitation, biofeedback and so) and medical therapy, high degree of bother (patients seeking definitive treatment) and positive stress and Q-Tip test. Our exclusion criteria were known neurological diseases affecting the bladder, normal urinary stress test, persistent positive urinary bacterial culture and active urogenital malignancy. After obtaining written informed consent from all patients, complete history including the beginning and severity of SUI, its course, and severity of urinary symptoms was collected. Physical examination was more focused in the abdominal and genitourinary exam with an examination of patients in lithotomy position for SUI, pelvic organ prolapse, and evaluating with stress and Q-tip test. Urinalysis and culture were performed, and post void residual volume was measured by ultrasonography. Patients asked to provide pads for test and a voiding diary. Pad test is defined as the 24-hour pad weight (24PWT). Patients were educated on how to perform the pad test, with clear instructions given about the use of similar pads, putting the whole pads in a zip-lock plastic bag the day before a visit, and bringing it along with a dry pad for gram weighing by the researchers. The degree of bother and severity of incontinence were also asked via International Consultation on Incontinence Questionnaire Short Form (ICIQ-SF) delivered to all

patients and filled in the presence of the researcher. We also evaluated all patients with uroflowmetry and complicated patients with complete urodynamic studies including filling and voiding cystometry with leak point pressure and urethral pressure profile. These included patients with severe irritative symptoms and urgency, history of neurologic diseases and stroke, long history of diabetes, previous SUI surgery and severe anterior prolapse.

Isolation and culture of ASCs

ASCs were isolated from abdominal subcutaneous adipose tissue. Adipose tissues harvested after obtaining informed consent under a protocol approved and supervised by Institutional Review Board (IRB). Tissue samples were transferred to the clean room facility (good manufacturing practice GMP and clinical grade stem cell manufacturing facility affiliated with Brain and Spinal Cord Injury Research Center) within a closed container at 4° C in RPMI 1640 (Roswell Park Memorial Institute medium, Gibco, USA) with 1% penicillin-streptomycin (Gibco, USA) up to 4 hours after tissue harvesting. The adipose tissue washed in phosphate-buffered saline (PBS; PAA, Germany) and small vessels and connective tissues dissected from the tissue. Then the adipose tissue was cut into small pieces and put into two 50 ml Falcon tubes. Tissue digestion performed by 0.5 mg/ml collagenase-NB6 (Serva, Germany) as a GMP grade enzyme which is considered for preparation of cell products (intended for clinical transplantation) followed by incubation at 37° C for 30 minutes. Digestion stopped by adding PBS-EDTA (in equal volume) and then the solution centrifuged (350 g, 10 min at room temperature). The stromal vascular fraction (SVF) re-suspended in PBS-EDTA and passed through 100 µm filter. Consequently, centrifugation was done at 400 g for 5 minutes and after counting the cells, SVF plated in DMEM (Dulbecco's modified eagle's medium, PAA, Austria) supplemented with 10% FBS-Pharma grade (PAA, Austria) and incubated at 37° C and 5% CO2. After 48 hours, the non-adherent cells washed and discarded and culture media replaced. The culture media renewed every 72 hours and after 90% confluence, the cells sub-cultured. After 2nd or 3rd subcultures, ASCs harvested by TryplE-Select which is manufactured on dedicated animal origin-free equipment (Invitrogen, USA) and prepared for injection in normal saline as transport media.

Microbiology tests

Microbiological tests were performed for aerobic, anaerobic, and fungi before, during and after cell processing and culture procedures.

Flowcytometry

Flowcytometric analysis performed to evaluate cell surface markers of ADSCs. Briefly, 1×10 5 cultured ASCs of 3rd subculture suspended in FACS-buffer (3% BSA in PBS) and then appropriate fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated primary antibodies added for 60 minutes at 4° C (CD44, 11b, 19, 34, 45, 105, 73, and HLA-DR; all from Abcam, USA). Consequently, labeled cells analyzed on a fluorescence-activated cell sorter (FACS, Partec).

Differentiation potential

Adipogenic differentiation

The cultured ASCs incubated in adipogenic medium (DMEM supplemented with 50 µg/ml indomethacin, 10 -7 M dexamethasone, and 50 µg/ml ascorbate-sodium 2-phosphate (all from Sigma) and maintained for around 25 days while the media renewed every 72 hours. Consequently, ASCs fixed by ethanol for 10 minutes and stained with oil red O.

Osteogenic differentiation

The cultured ASCs incubated in osteogenic medium (DMEM supplemented with 10 mM ß–glycerol phosphate, 50 µg/ml ascorbate sodium 2-phosphate and 10-7 M dexamethasone, 1% antibiotic/antimycotic (all from Sigma) and maintained for around 25 days while the media renewed every 72 hours. Consequently, ASCs fixed by ethanol for 10 minutes and stained with alizarin red.

Concomitant transurethral and transvaginal-periurethral injection of ASCs

All patients underwent a diagnostic cystourethroscopy just before the operation. There was a possibility to once again check urethral hypermobility, sphincter competence, coaptation, and stress test. The patients treated between 18 August 2012 and 15 December 2012. Cell injection (1,180,000 cells/ml, 10 ml) performed in the lithotomy position, under local anesthesia with lidocaine gel (%2) injection in urethra 10 minutes prior to cell injection plus periurethral anesthesia by 5 cc lidocaine (%2) injections to periurethral space. Urethroscopy was done with 19 F sheath and 5F needle. 2/3 of cells injected equally in 2 and 10 clock position in 0.5 cm depth of mid-urethra transurethrally. Later, with the slight downward pushing of the sheath to produce a marker bump on the vaginal

mucosa, the needle again entered, and the remaining of the cells (1/3) were injected to periurethral space transvaginally under concurrent urethroscopic view. The patients asked to cough just after the injection, to make sure the injection was properly administered. Post injection endoscopic control performed for all patients to rule out needle entrance in urethra, then bladder emptied. No indwelling urethral catheter inserted for the patients after injection therapy, and they encouraged voiding at their convenience. Postoperatively, the patients discharged on the operation day, provided that they had no voiding problems. Certainly, patients were put on antibiotic prophylaxis before the procedure and a few days after. Residual urine measured. They asked to take 24 hours voiding diary, 24-hour pad test and any voiding problems. Urodynamics and uroflow studies and International Consultation on Incontinence Questionnaire (ICIQ) results were taken on definite intervals. The outcome criteria were collected at 2, 6 and 24 weeks' intervals. These intervals give a good scheme of the short term results of the procedure. Although many factors considered for evaluation of incontinence as the outcome measure, the three main focuses for statistical analysis were Maximum Flow Rate (Qmax) pre and 2 weeks post procedure, 24 hours' pad test results on intervals, and ICIQ results on predefined intervals. The pad test was exactly performed the same as the first visit and was scheduled to be undertaken by patients a day or two prior to following up visit. ICIQ Statistical analysis performed using paired T-test for Qmax means, and Repeated Measures ANOVA for pad test and ICIQ results which had four consecutive variables to compare. The data analyzed with SPSS software version 18.

Results

Characterization of ADSCs

ASCs were negative for CD11b, 19, 34, 45, HLA-DR, and positive for CD105, 44, and CD73. Means of viability and purity were 97.8 % and 93.5% respectively (Figure 1).

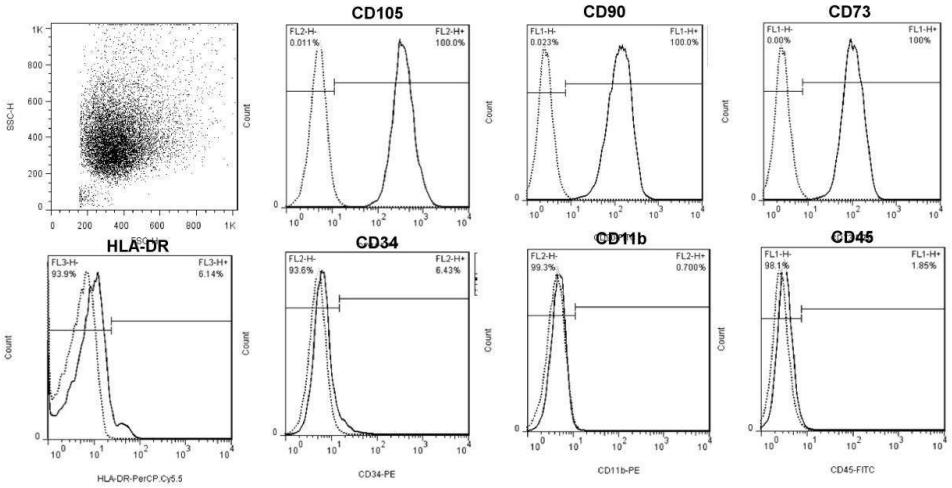

Figure 1. Flowcytometric analysis of ASCs at 3rd subculture

Differentiation potential

We evaluated the differentiation potential of ASC isolated by staining with Oil Red O (adipogenic), Alizarin Red (osteogenic) (Figure 2).

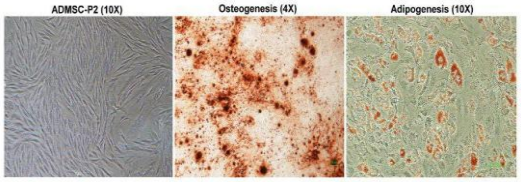

Figure 2. Adipogenic (A) and osteogenic (B) differentiation of ASCs

All ten cases completed the study. The mean age of the participants was 45.8±8.7 years, the mean height was 162.4±5.5 cm, and the mean weight was calculated at 71.8±8.6 kg. Past history of urinary incontinence varied from two to more than five years. Of the whole group, 6 (60%) had pelvic organ prolapsed, and 1 (10%) had undergone hysterectomy. Urinary incontinence significantly decreased through the first two weeks after injection therapy. This could be shown by comparing the results of pad test, preoperative, 2, 6 and 24 weeks' measurements. Comparing the four variables' means showed an overall significant difference between the repeated measures of pad test (Wilks' lambda=0.79; *P*<0.001). Testing within-subject contrast showed a statistically significant difference between pad test preoperatively and 2 weeks (*P*<0.001), and also between 6 weeks and 24 weeks (*P*=0.018) postoperatively, but not between 2 weeks and 6 weeks (*P*=0.146). This might imply the first bulking effect of the injected substance, a gradual decrease in its bulk, and a third re-growth of stem cells to mend the weak urinary sphincter. Figure 3 (Plot A) shows the plot of the pad test on several intervals. Scores of ICIQ-SF showed almost similar findings. Here again, the overall comparison showed a significant difference between pre and postsurgical scores (Greenhouse-Geisser sum of square=317.2; *P*=0.000). Testing within subject contrast here showed a statistically significant difference between presurgical scores and 2-week scores (*P*=0.000) but not for the next two consecutive measures. The results are depicted in Figure 3 (Plot B). Not only the goal of continence achieved, but also an improvement in maximum flow rate 24 weeks after surgery was encountered, which also was statistically significant (Qmax means 32.6 vs. 35.7; *P*=0.002). Complications of the procedure with one patient experiencing slight voiding difficulty, which needed catheterization, no urinary tract infections or gross hematuria, occurred. Residual urine volume was insignificant (except in one case). There was no case of chronic retention.

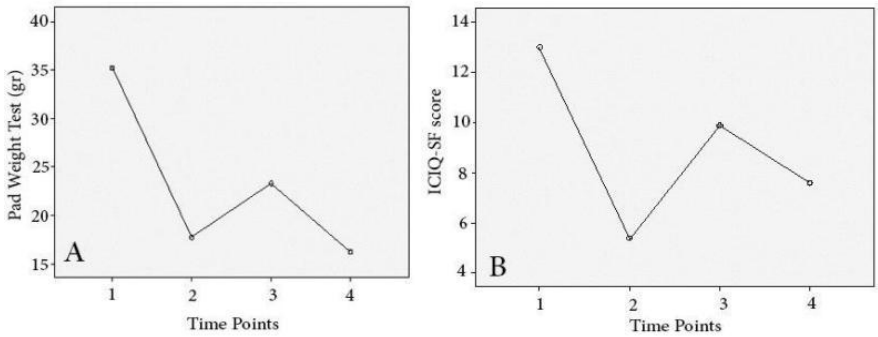

Figure 3. Pad Test Plot (A), ICIQ-SF score plot (B).1=baseline pad test results, 2=2 weeks' pad test results,3=6 weeks' pad test results, 4=24 weeks' pad test results

Discussion

As mentioned before, complications and poor long-term efficacy of periurethral injection of bulking agents, lack of success in pharmaceutical treatment and complications of invasive procedures such as sling surgery leads to alternative treatments, especially those which are able to restore natural continence physiology (27,29). Therefore, cell therapy is presently considered as having great therapeutic potential. Cellular therapy has recently proved to be a new treatment modality, different techniques for various indications has emerged (7,27,30-33). Adipose-derived stem cells have been the one to be widely used in current years. There have been some studies reporting successful use of them in human diseases (34,35). Additionally, ASC has been successfully used for the treatment of SUI in animal models, but there are a few studies reported in human (36,37). Although the results have been promising, small case numbers and choosing the male population with urinary incontinence necessitate complementary studies. In another study, injection of autologous muscle stem cells for the treatment of SUI and improvement of sphincter mechanism has been reported with successful results (38). Another group studied periurethral injection therapy of autologous fat but with moderate outcomes (7). One of the last attempts has been injections of autologous total nucleated cells (TNCs) along with

platelets to the urethral region in females with SUI (32). Bone marrow mesenchymal stem cell (MSC) transplantation is another method of cell therapy (33). However, cell therapy is still a demanding task. Using MSCs has problems like the invasive procedure of harvesting, long and difficult culturing (29) by the way, adipose tissue has plenty of multipotent stem cells, near 100-fold of bone marrow, and is quite easily harvested. So adipose tissue is an attractive source for tissue engineering (27,29). The current study is distinct because a great number of cases, using adipose derived stem cells for injection therapy and choosing female population for evaluation. There seems to be a complex and staged mechanism for treating SUI in functional agents like adipose derived stems cells injection therapy. In the first few weeks, there would be a bulking effect of the injected suspension to the periurethral area. This addresses the considerable improvement of incontinence in 2-week results of the study, during which patients were feeling completely cured and continent. Both on pad test and ICIQ results and plots, one can observe this tremendous effect. Between 2 and 6 weeks, the results are not that satisfactory. We observed that the ICIQ and pad test results weaned back, and the plots took a reverse slope. This window period was predictable, as any natural bulking agent will dissociate and solve away, so losing the acute bulking nature. By the way, it launches for a different future, as stem cells are preparing to perform their main duty. In the third phase, the results which we observed in 24 weeks after the injection became promising again. This was proved statistically by the results of pad test and could be observed on the last slope on plots. Here is when stem cells proliferated, recruited and some might have been differentiated to functional contractile cells, as it was shown by pathology in previous animal studies (30). This foretells the same mechanism or even pathology in human studies, although we could not biopsy the injected region for ethical reasons. The procedure had no important complications, as most of the minimally invasive injection therapies. This could be a bargaining chip over more invasive surgical procedures like bladder neck suspension, although data for any comparison is currently lacking .This study showed that the autologous adipose-derived stem cells (ASCs) transplantation to the periurethral region is a safe and effective in short term treatment option for stress urinary incontinence in the female. More studies with a large number of case, longer follow-up times and pathologic confirmation might be needed to popularize the treatment method, and confirm that stem cells are truly making a change.

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