

A Non-invasive Method to Evaluate the Efficacy of Human Myoblast in Botulinum-A Toxin Induced Stress Urinary Incontinence Model in Rats

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Received July 2012
Accepted December 2012

Purpose: To develop a simple non-invasive method to assess the efficacy of a cell based therapy for treating stress urinary incontinence (SUI).

Materials and Methods: In this study, skeletal myoblasts were used as candidate therapy to reverse SUI. The SUI model was created in rats using periurethral injection of botulinum-A toxin injection. Two weeks later, the rats were administered saline and the level of continence in each botulinum-A toxin treated and control animals was assessed by the extent of voiding using metabolic cages. To determine the efficacy of myoblasts to reverse SUI, botulinum-A toxin treated incontinent rats were injected with either cultured human skeletal myoblasts or with buffered saline (sham control). Two weeks post implantation, the extent of continence was evaluated as mentioned above.

Results: The difference in void volume between botulinum-A toxin -treated and control rats were significant. Histological analysis of the urethra showed remarkable atrophy of the muscular layer. A significant reversal ($P = .025$) in the volume of voiding was observed in cell-implanted rats as compared to sham injected rats. Histological analysis of the urethra implanted with myoblasts showed recovery of the atrophied muscular layer in comparison to sham control. Immunofluorescence analysis of the cell injected tissues confirmed the presence of human myoblasts in the regenerated area.

Conclusion: This simplified method of in vivo testing can serve as a tool to test the efficacy of new therapies for treating SUI.

Keywords: muscle; botulinum toxins, type A; urinary incontinence; stress; urethra; rat; disease model.

INTRODUCTION

It has been reported that more than 200 million people are afflicted with urinary incontinence (UI) worldwide⁽¹⁾ and nearly half of them have symptoms of stress urinary incontinence (SUI). SUI severely impacts quality of life and its etiology is considered to be multifactorial. Current therapies for SUI do not treat the underlying causes and often involve the introduction of foreign materials such as silicone particles, carbon beads and bovine collagen.⁽²⁾ However, the efficacy of such treatment declines with time, and repeated injections are required.⁽³⁾ Other disadvantages of these include periurethral abscess, chronic inflammation and obstruction of the lower urinary tract, severe voiding dysfunction, and pulmonary embolism.⁽⁴⁾ Treatment of SUI with conventional surgical sling procedure sometimes results in postoperative voiding difficulty with a limited cure rate due to intrinsic sphincter deficiency.⁽⁵⁾

The potential of cells such as skeletal muscle derived myoblasts and stem cells like adipose tissue-derived stem cells (ADSC) and bone marrow derived mesenchymal stem cells to reverse SUI have been studied extensively.^(6,7)

Although none of the existing animal models completely simulate the human situation, nonetheless, animal models are widely used to understand the pathophysiology of SUI and enable preclinical testing of potential treatments.⁽⁸⁾ Several SUI animal models such as nerve injury,^(9,10) urethral cauterization,⁽¹¹⁾ pubourethral ligament injury, urethrolisis,⁽¹²⁾ and botulinum-A toxin induced chemical denervation⁽¹³⁾ models have been developed to understand different aspects of urinary continence mechanism.

In SUI, unintentional urine leakage occurs as a result of a behavioral condition. Since animals cannot indicate their intent, the assessment of SUI in animal models therefore involves functional surrogates of urethral resistance to leakage.⁽¹⁴⁾ Methods such as Urethral Closure Pressure testing, Sneeze testing and Leak point Pressure (LPP) testing etc are some of the methods that are used for assessing SUI in animal models.⁽¹⁵⁾

Cannon and colleagues had demonstrated the formation of new skeletal muscle fiber following an injection of skeletal muscle cells (myoblasts) in the urethra.⁽¹⁶⁾ Autografting of muscle precursor cells in a murine model of urethral sphinc-

ter injury has also been reported.⁽¹⁷⁾ The results demonstrated that this procedure may accelerate sphincter muscle repair by producing a significant increase in the diameter and number of myofibers, suggesting that these cells could serve as a potential therapeutic approach to treat urethral sphincter insufficiency.

The aim of our present study was to develop a simplified noninvasive method to test the efficacy of treatment for SUI. Using cultured human myoblasts as a candidate, we have tested its efficacy in a botulinum-A toxin induced SUI animal model. In this study, cultured human myoblasts were injected periurethrally and efficacy of the implanted cells to reverse SUI was analyzed following intraperitoneal administration of saline. The volume of urine voided was compared in rats injected with and without cells. Histological tests were performed to check the morphology of the urethra and immunohistochemical analysis was done to confirm the presence of the implanted cells.

MATERIALS AND METHODS

Materials

Myoblast culture media (SKGM-2 bullet kit) was purchased from Lonza, USA. The Dulbecco's Modified Eagle's Medium (DMEM) and all other cell culture reagents were purchased from Sigma, USA. Plastic ware for cell culture was obtained from NUNC, USA. Calcium phosphate transfection kit was purchased from Promega, USA, and antibodies (desmin and myosin 1A-heavy chain) were from Abcam, USA. The cDNA of the GFP constructed into the lentiviral vector PrlSinDeco, was a kind gift from Dr. Wei Li (Department of Dermatology, University of Southern California, USA).

Animals

Wistar rats were bred in-house. All animals were handled in accordance with the CPCSEA guidelines for the welfare of laboratory animals practices laid down by the Government of India. The study was approved by the Institutional Animal Ethical Committee (IAEC).

Myoblasts Isolation, Culture and Characterization

Skeletal muscle biopsies were collected from patients undergoing elective surgery after receiving informed consent

Table. Volume of urine voided by control and treated rats in metabolic cages.

Treatment Group	No. of Animals	Volume of Urine Voided (mL)
Saline Control	3	0.9 ± 0.2
Botulinum A Toxin	12	4.4 ± 0.5
Sham Control	3	4.5 ± 0.6
Myoblasts Treated	6	1.3 ± 0.4

and approval from an independent institutional ethics committee. Biopsy samples were transported to the lab and were processed under aseptic condition. Briefly, the biopsies were rinsed in Hanks buffered saline solution (HBSS) and their surface was decontaminated by immersing in Povidone-Iodine (Win Medicare, India) for 1-2 min. The tissues were further incubated for 20 min serially in 10×, 5× and 1× concentration of Ampicillin-Amphotericin-Streptomycin (AAS) solution (Gibco). The tissues were chopped into small pieces and digested in a solution containing a mixture of 1.2 units of dispase and 4 mg/ml of collagenase IV (1:1), for 30 min at 37°C with intermittent shaking. The resulting tissue suspension was passed through 70µm strainer (Becton Dickinson, USA) and centrifuged for 5 min at 1200 rpm. The cell pellet was then re-suspended in myoblast growth media (SKGM-2 bullet kit from Lonza, USA) supplemented with 10% fetal bovine serum (Lonza, USA) and plated in tissue culture dishes and incubated at 37°C in a humidified atmosphere containing 5% CO₂. To obtain an enriched myoblast population, the unattached cells in the dishes were transferred after 48 h to collagen-I (Sigma) coated plates (100 ng/mL). Media was changed every third day in the coated dishes till the cells reached 70-80% confluency. The cultured myoblasts were purified by MACS® separation (Milteny Biotec, USA) using anti-human desmin antibody.

The identity of the isolated cells was further confirmed by staining with the above desmin antibody. Differentiation of the purified myoblasts to myotubes was induced by culturing highly confluent myoblasts (> 80% confluency) in differentiation media containing DMEM and 2% horse serum (Lonza, USA) for two weeks. Myoblasts were identified by standard immunofluorescence method. Briefly, cells were fixed with cold acetone for 20 min followed by incubation with mono-

clonal anti-human desmin antibody (1:50). Positive cells were identified by counterstaining with FITC-conjugated anti-mouse antibody (1:500) (BD Bioscience, USA). Differentiated myoblasts were identified after incubation with monoclonal myosin heavy chain (MHC) antibody (1:100), followed by Alexa-fluor-568 conjugated secondary antibody. Total cells in each field were identified by 4'-6-Diamidino-2-phenylindole (DAPI) (Sigma) staining and visualized using fluorescence microscope (Observer.Z1.Carl Zeiss, Germany) Transduction of GFP into Myoblasts Using Lentiviral Vector The lentivirus-derived vector pRRLsinhCMV was inserted with eGFP cDNA using EcoRV. This construct was used to co-transfect 293T cells together with packaging vectors pCMVΔR8.2 and VSVG. Typical viral titers were $1-7 \times 10^6$ transduction units/ml as measured by previously described method.⁽¹⁸⁾ The cell infection efficiency was 66% (data not included) as monitored by the percentage of cells positive for GFP expression using FACS analysis [FACS Calibur (E3851), (Beckton Dickenson)].

Creation of SUI Model in Rats

The SUI model was created in rats by using the method described by Takahashi and colleagues.⁽¹³⁾ Briefly, twelve rats aged between 4-6 weeks were anesthetized with ketamine (80 mg/kg) and xylazine (40 mg/kg) intraperitoneally. Physiological saline containing botulinum-A toxin (Allergan, India), (7U/100 µL) was injected periurethrally at the mid urethra, which was located at the level of the symphysis pubis. Three rats were similarly injected with only saline (control). The rats were kept in individual cages and had free access to water and food.

Incontinence Testing

Two weeks after the botulinum toxin treatment, 10 mL of normal saline solution warmed to 37°C was injected intraperitoneally into each rat and the animals were housed individually in metabolic cages (Tecniplast, Italy). Bladder function was assessed by measuring urine output at the end of 15 minutes after saline injection. Saline was injected thrice in each animal at an interval of 30 minutes, and the average volume voided by each animal was calculated.

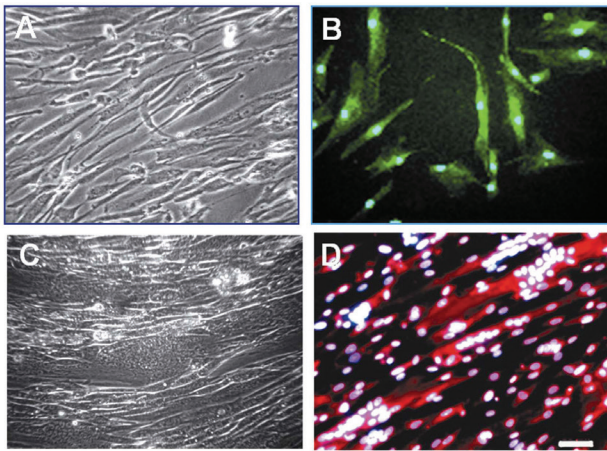


Figure 1. Phase contrast images of myoblasts (A) and (C) myotubes. Fluorescence images of myoblasts stained with anti-desmin antibody (B) and myotubes stained with anti-MHC-1A antibody (D). Scale bar represents 50 μm .

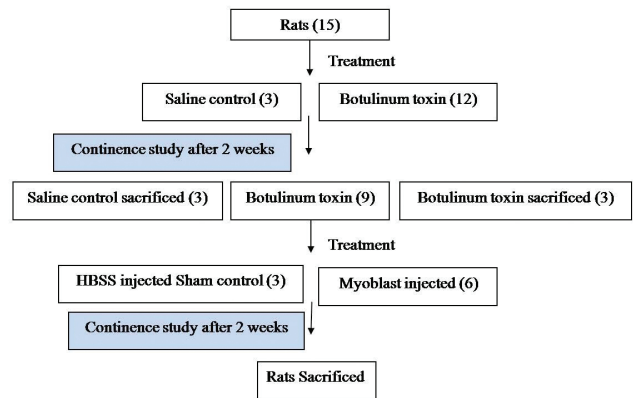


Figure 2. Diagrammatic representation of the experimental design.

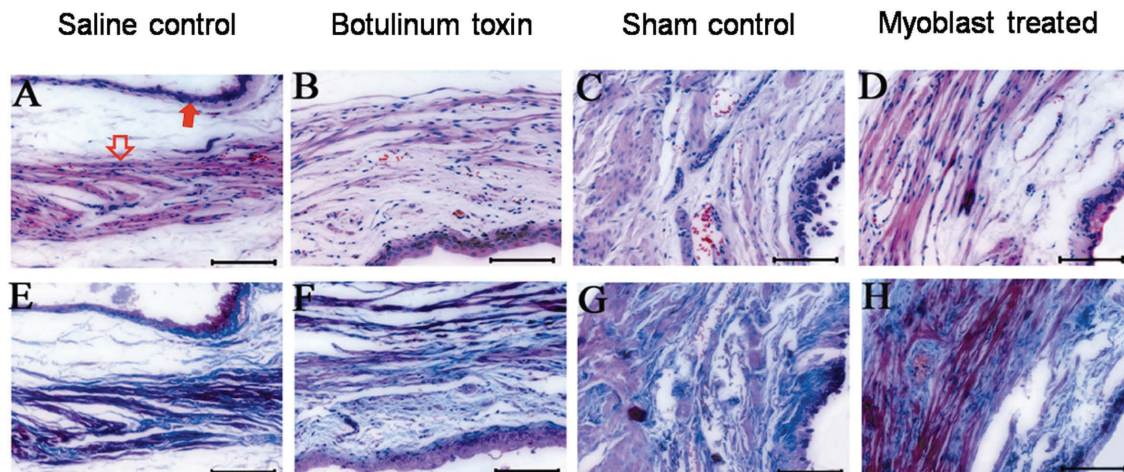


Figure 3. Representative sections of rat urethra stained with hematoxylin eosin (top lane) and Masson's trichrome stain (bottom lane). Scale bar represent 100 μm .

Periurethral Implantation of Labeled Cells

To study the efficacy of implanted myoblasts on SUI, the incontinent rats created as mentioned earlier were immunosuppressed with cyclosporine A (5mg/kg of body weight) starting 2 days prior to the implantation of cells till the end of the study.⁽¹⁹⁾ Six rats were injected with GFP positive myoblasts (8×10^6 cells) suspended in HBSS on either side of urethra, and three animals were similarly injected with HBSS buffer alone (sham control). After two weeks, the continence test was repeated in the sham control and cell injected test ani-

mals. The animals were euthanized and the urethra was excised for histological and immunohistological analysis.

Histological Analysis

For histological analysis, the excised mid-urethra was fixed in 10% buffered formalin, and embedded in paraffin blocks and sectioned into 5- μm thick slices. These were de-paraffinized and hydrated with water. Sequential sections were stained with hematoxylin and eosin or Masson trichrome according to the manufacturer's protocol (Sigma-Aldrich, USA).

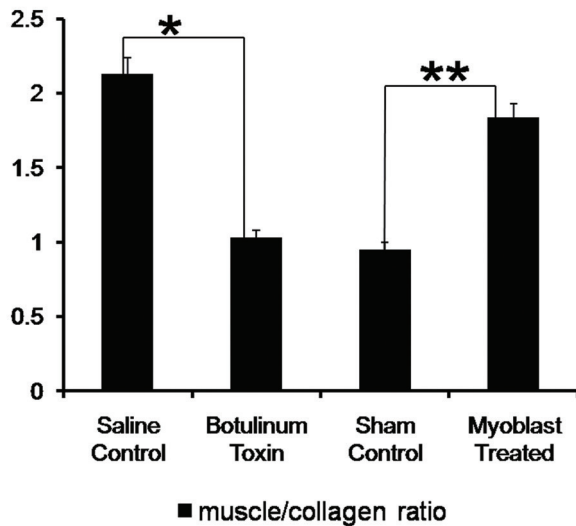


Figure 4. Ratio of muscle/collagen in the urethral wall of treated and untreated rats. * $P = .002$ and ** $P < .001$.

The effect of treatment on the muscular layer of the urethra was evaluated using light microscopy and photographed. The mean thickness of the four regions of external urethral sphincter (EUS) comprising striated muscles, near the two diagonal lines was evaluated in detail in each rat using Zen software (Carl Zeiss, Oberkochen, Germany).

To prevent variations in Masson's trichrome staining, all samples were stained simultaneously. Images from the entire sections were acquired under light microscope (Observer. Z1, Carl Zeiss, Oberkochen, Germany). Following Masson's trichrome staining of the sections, cells in blood vessels, smooth muscle layer and rhabdosphincter layer, stained red while collagens stained blue. The multiple images were analyzed using the software Zen (Carl Zeiss, Oberkochen, Germany) which automatically distinguished regions stained with different colors and measured the area of muscle and collagen to yield muscle/collagen ratio.

Immunohistochemical Analysis of Myoblasts

Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μm thickness. These sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the deparaffinized sections in citrate buffer in a microwave for 35 seconds followed by cooling at room temperature for 20 min. The sections were further incubated in 1% bovine serum albumin (BSA) in PBS for 1 hr at room temperature followed

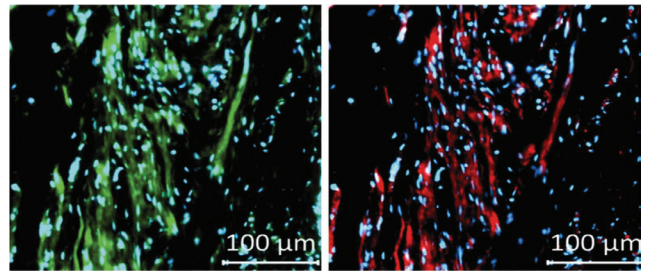


Figure 5. Fluorescence images of human myoblast implanted rat urethral wall. A. GFP positive human myoblasts are identified as green cells. B. The same section counter stained with anti-human desmin antibody and counter stained with Alexa-fluor-568 secondary antibody. Positive cells are visualized as red cells. Total cells in the field are identified after staining with DAPI.

by incubation with mouse anti-desmin monoclonal antibody, (ABCAM Biotech, USA) at 4°C overnight. Desmin positive cells were detected by counter staining with Alexa-fluor-568 secondary antibody (Molecular Probes, Invitrogen, Oregon, USA). Total cells in the sections were visualized by staining with DAPI. Green and red fluorescent cells were visualized under a fluorescence microscope (Observer. Z1, Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Histological data are reported as mean (median). Microsoft Excel was used for statistical analysis. Mann Whitney U test was used to determine the significance of difference in voiding as well as morphometric data seen between rats implanted with human myoblasts and control. Statistical significance was determined at P values < 0.05 .

RESULTS

Cultured Myoblasts Characterization

Human myoblasts were highly enriched following MACS® separation. Under phase contrast microscope, cultured myoblasts are spindle shaped (Figure 1A) and expressed myoblast specific desmin (1B). In presence of low serum, the cultured myoblasts differentiated to form multi-nucleated myotubes (Figure 1C) that expressed myosin heavy chain (MHC-1A).

Evaluation of Continence

A pilot study using 2.5, 5, 7 and 10 U of botulinum-A toxin was conducted in wistar rats to identify the effective concentration of botulinum-A toxin needed to cause chemical denervation of the urethra. Based on the results of the pilot study, 7 U was identified as the optimal dose required for inducing incontinence. A diagrammatic representation of the experimental design is shown in Figure 2. As shown in Table, a significant increase in the volume of micturition was observed in all animals injected with botulinum-A toxin as compared to control ($P = .009$).

The effect of myoblast implantation/HBSS injection (sham control) in the above SUI induced rats was assessed using the same procedure as mentioned earlier. A significant difference was observed in the volume of urine voided between sham and myoblasts injected animals ($P = .025$). 84% of rats injected with myoblasts regained continence within two weeks as compared to 0% in HBSS injected animals.

Histological Analysis

Histological examination of the urethra stained with hematoxylin and eosin revealed a typical morphology in saline injected control rats (Figure 3A). The tissue showed several layers, starting with the epithelial layer (filled arrow) then the underlying lamina propria consisting of a layer of smooth muscle cells and the external urethral sphincter (EUS) made of a thick layer of striated muscle (open arrow). Mid-urethral cross-sections showed striated and smooth muscle fibers circumferentially around the urethra. The mean thickness of the four regions of striated muscle, near the two diagonal lines was evaluated in each rat. The morphological images revealed that the striated muscles significantly atrophied at 2 weeks after botulinum-A toxin administration (Figure 3B). The thickness of EUS was 82.4 (80) μm at two weeks in botulinum-A toxin injected urethra as compared to 172.3 (162) μm in control rats ($P < .0001$). Following myoblast implantation, the thickness of the muscle layers increased to 192.7 (205) μm as compared to sham injected control which was 91.3 (94) μm ($P < .0001$).

The distribution of muscle to collagen in the EUS area of Masson's trichrome stained sections was captured as a ratio using the Zen1-Observer software. Four to six random areas in each section were analyzed under 200 \times magnification. As

shown in Figure 4, the ratio of muscle to collagen content in botulinum toxin injected rats reduced to 1.03 (1.0) as compared to saline injected control rats 2.1 (1.9), $P = .002$. Sham control group presented a muscle/collagen distribution similar to the botulinum toxin injected urethral wall 0.94 (0.8), $P = .1$. Following myoblasts implantation, however the muscle content significantly increased as compared to sham control 1.85 (1.85), $P < .0001$. Figure-3 (E-H) shows representative Masson's trichrome stained sections at different time points. Presence of GFP positive cells in the paraffin sections of urethra of rats implanted with human myoblasts indicated the presence of implanted myoblasts in the urethra (5A). The same section on counter staining with human desmin antibody confirmed the presence of human cells (5B). The GFP positive cells had formed myotubes and were seen to be aligned along the rhabdosphincter. Significance was observed between saline and Botulinum-A toxin injected ($P = .009$) and sham control and myoblast treated ($P = .025$)

DISCUSSION

Clinical treatments for SUI include conservative techniques, pharmacologic therapy, and surgical procedures. In the clinical condition, sophisticated urodynamics and other related tests are performed for diagnosis and treatment. To test the efficacy of new surgical techniques or pharmacologic targets it is still necessary to use animal models of SUI. The existing techniques to evaluate efficacy of a therapy in SUI animal models are labor intensive and require specialized instruments. Besides, existing animal testing methods require anesthesia to immobilize the animal in addition to invasive and non-survival studies. The main purpose of the present study was to develop a simplified noninvasive method to evaluate the efficacy of a therapy such as myoblast therapy in a SUI model.

Takahashi and colleagues had earlier demonstrated that periurethral injection of botulinum-A toxin induced chemical denervation lead to a significant decrease in LPP, and remarkable shrinkage of the smooth muscle layer and striated sphincter.⁽¹³⁾ As compared to pudendal nerve transection⁽²⁰⁾ and electrocauterization^(21,22) methods used to impair urethral sphincter, botulinum-A toxin induced urethral muscle sphincter impairment does not involve an abdominal inci-

sion. We had chosen this noninvasive model to create SUI. While 10 U botulinum-A toxin was used in Takashi's study, our preliminary studies indicated 7 U was optimal for SUI creation. This could be attributed to the difference in the animal strain being used in the study. In the incontinence model developed by Lin and colleagues using vaginal balloon dilation method, only 46% of animals were deemed incontinent after the procedure⁽²⁶⁾ whereas with botulinum toxin induced SUI model 100% of animals became incontinent. Disadvantage of both models is the spontaneous restoration of continence with time.

To determine urethral resistance in animal models of SUI, several methods mimicking a variety of clinical urodynamic tests have been developed. One of the most widely used methods to evaluate urethral resistance in rats is LPP.^(13,21,24,25,27) In these animal models, the intravesicular pressure is evaluated by urethral or suprapubic catheter. However, since LPP evaluation in rats can trigger micturition and urethral catheter may increase the urethral resistance, a well-trained investigator is required to eliminate confounding variables. Our method of evaluation which is also based on urethral resistance to leakage does not involve the use of a catheter and overcomes some of these challenges facing LPP test.

Several studies have shown that direct injection of muscle derived cells improve urethral sphincter contraction and contribute to continence in animal models of SUI.^(23, 24) Periurethral injection of muscle derived cells improved the LPP in a denervated female rat model of SUI,⁽²⁰⁾ and in rats that showed intrinsic sphincter deficiency following radical prostatectomy.⁽¹¹⁾

Using human myoblasts as a candidate we have demonstrated the efficacy of periurethrally implanted muscle cells by analyzing the urine output following cell implantation. Our results corroborate with LPP data observed in other similar studies.^(21,24,25,27) Kim and colleagues demonstrated the feasibility of using muscle derived human cells in nude denervated rat SUI model.⁽²⁵⁾ A decrease in LPP from control levels was observed in the sham group following denervation of urethra. As compared to the sham group, the group reported restoration of LPP in the rats injected with muscle derived cells. Using a botulinum toxin induced SUI rat model, we similarly observed a significant increase in micturi-

tion in botulinum toxin injected rats as compared to control group ($P < .05$). This increased micturition could be a result of decreased LPP. The authors' observation correlates with our data wherein the recovery of continence was observed following myoblast implantation when compared to sham group ($P < .05$).

The reversal of incontinence observed in our study could be attributed to the presence of implanted cells as confirmed by positive staining with human muscle specific desmin antibody. While the implanted cells might have contributed directly to the formation of the skeletal muscle, its paracrine effect on resident stem cells to stimulate new muscle formation cannot be ignored. Some of the structural changes observed in the intrinsic structure of the urethra affected with SUI was observed in our histological analysis.^(28,29) Our data demonstrated that urethral dysfunction induced by botulinum toxin was accompanied by decreased muscle content and/or increase in connective tissue deposition which was reversed following myoblast implantation.

CONCLUSION

The simplified non-invasive technique that was employed in this study to assess continence can be used as a screening method to check for efficacy of potential candidates for SUI treatment.

ACKNOWLEDGEMENTS

We acknowledge the encouragement and support of Reliance Life Sciences Pvt. Ltd to carry out the research work (www.rellife.com). We thank Dr. Harinarayana Rao, Dr. Akash Shinde and other members of LARS and Tissue Engineering Group for their support.

CONFLICT OF INTEREST

None declared.

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