# Treatment of stress urinary incontinence with low-intensity extracorporeal shock wave therapy in a vaginal balloon dilation induced rat model

**Alex K. Wu, Xiaoyu Zhang, Jianwen Wang, Hongxiu Ning, Uwais Zaid, Jaqueline D. Villalta, Guifang Wang, Lia Banie, Guiting Lin, Tom F. Lue**

Knuppe Molecular Urology Laboratory, Department of Urology, School of Medicine, University of California, San Francisco, CA, USA

*Contributions:* (I) Conception and design: TF Lue, G Lin; (II) Administrative support: TF Lue, L Banie, G Lin; (III) Provision of study material or patients: TF Lue, AK Wu, G Lin; (IV) Collection and assembly of data: AK Wu, X Zhang, J Wang, H Ning, U Zaid, JD Villalta, G Wang ; (V) Data analysis and interpretation: AK Wu, X Zhang, J Wang, U Zaid, JD Villalta, G Lin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*Correspondence to:* Tom F. Lue, MD. Department of Urology, University of California, San Francisco, 400 Parnassus Ave., Ste A-633, San Francisco, CA 94143-0738, USA. Email: tom.lue@ucsf.edu.

**Background:** To investigate the outcomes and mechanisms of low-intensity extracorporeal shock wave therapy (Li-ESWT) on stress urinary incontinence (SUI) in a vaginal balloon dilation (VBD) rat model.

**Methods:** Thirty Sprague-Dawley rats were randomly grouped into normal controls, VBD only, and VBD with Li-ESWT. Li-ESWT was administered twice per week for 3 weeks. Afterward, all 30 rats were assessed with functional and histological studies. To explore the acute effect of Li-ESWT, another 25 rats, given intraperitoneal 5-ethynyl-2-deoxyuridine (EdU) at birth, were treated with Li-ESWT followed by assessment of vascular endothelial growth factor (VEGF) expression and endogenous progenitor cells distribution at 24 hours or 1 week after the last Li-ESWT therapy. Additionally, rat myoblast L6 cells were used for myotube formation assay *in vitro*.

**Results:** Functional analysis with leak-point pressure (LPP) testing showed that rats treated with Li-ESWT following VBD had significantly higher LPP relative to those receiving VBD only (44.8±3.2 versus 27.0±2.9 cmH$_2$O, P<0.01). Histological examinations showed increased urethral sphincter regeneration in Li-ESWT group. The rats treated with Li-ESWT also had increased vascularity, which was confirmed by immunohistochemistry of rat endothelial cell antigen, while reverse-transcriptase polymerase chain reaction (RT-PCR) showed VEGF expression was significantly enhanced. Additionally, there were significantly increased EdU+ cells in Li-ESWT treated rats at 24 hours. *In vitro*, Li-ESWT promoted myotube formation from L6 cells.

**Conclusions:** Li-ESWT ameliorated SUI by promoting angiogenesis, progenitor cell recruitment, and urethral sphincter regeneration in a rat model induced by VBD. Li-ESWT represents a potential novel non-invasive therapy for SUI.

**Keywords:** Low-intensity Extracorporeal Shock Wave Therapy (Li-ESWT); stress urinary incontinence (SUI); vaginal balloon dilation (VBD); leak-point pressure (LPP); 5-ethynyl-2-deoxyuridine (EdU)

## Introduction

More than 25% of women suffer from stress urinary incontinence (SUI) in the United States and the annually cost related to management of this condition is high (1,2). Current treatments for SUI, including oral medications, urethral bulking agents, and urethral sling surgeries, provide limited efficacy or can incur short-term and long-term complications (3-5). Moreover, these therapies treat only the symptoms, not the underlying pathology. Therefore, novel treatments to restore normal urethral function are urgently needed (6).

Stem cell (SC) therapy for SUI was first published in 2002 (7) and has since advanced to several clinical trials (6). In 2010 we firstly published a preclinical study in which adipose-derived stem cells (ADSCs) were used for SUI therapy (8). As ADSC can be more easily, abundantly, and safely obtained than other SC types, it has become the choice cell type in many recent studies (6,9). Clinically, the use of SC therapy is still very limited due to safety and efficacy concerns. Recently, low-intensity extracorporeal shock wave therapy (Li-ESWT) has been applied in regenerative medicine to stimulate skeletal muscle growth and accelerate repair (10). Li-ESWT has been shown to mobilize endogenous SCs to injury sites (11,12), and to activate local stem/progenitor cells for the treatment of various urological disorders including erectile dysfunction (ED) and chronic pelvic pain syndrome (13-16). Recently, we also demonstrated that Li-ESWT is able to activate penile endogenous SCs (17,18) and promote myogenesis *in vitro* (19). In addition, Li-ESWT therapy has also been shown to promote host tissue angiogenesis and neuroregeneration (13,20,21).

Multiple animal studies examining tissue grafts, skin flaps, and myocardium have shown the increased angiogenesis and re-vascularization effects of Li-ESWT (22-24). In a rat diabetic ED model (25), we also found that Li-ESWT was able to significantly reverse diabetes-associated deficits in penile muscle, endothelium, and nerve contents. In addition, we obtained evidence that Li-ESWT was able to mobilize endogenous SCs to injury sites—a phenomenon previously observed in hind limb ischemia animal models (11,12). Li-ESWT represents a potential safe and effective treatment for various trauma-associated disorders, including SUI related to birth trauma.

## Methods

### Animals and overview

All experimental protocols were approved by the Institutional Animal Care and Use Committee at our institution. Thirty female Sprague-Dawley rats (12 weeks) were obtained from Charles River Laboratories (Wilmington, MA, USA), and randomly divided into 3 groups including normal controls (10 rats), vaginal balloon dilation (VBD) only (9 rats), and VBD with Li-ESWT treatment (11 rats). VBD and subsequent ovariectomy was performed as previously described (26,27). Briefly, after rats were anesthetized, VBD was performed via vaginal insertion of a 16-Fr latex Foley catheter, with subsequent filling of the catheter balloon to 4 mL of fluid. The balloon was left in place for 4 hours to simulate prolonged labor. One week later, the rats were again anesthetized; and both ovaries were excised with a midline incision. Li-ESWT was administered twice per week for 3 weeks under anesthesia. One week wash-out following completion of Li-ESWT therapy, approximately 5 weeks after initial VBD, leak-point pressures (LPP) were measured for functional evaluation, and then the rats were sacrificed with the urethras harvested for histological analysis.

An additional set of twenty-five 8-week-old female Sprague-Dawley rats that had been injected with 5-ethynyl-2-deoxyuridine (EdU, Invitrogen, Carlsbad, CA, USA) at birth as previously described, were divided into 3 groups including normal controls (6 rats), VBD only (7 rats), and 1 week of Li-ESWT (twice a week) following VBD with sacrifice at 24 hours (6 rats) or 1 week (6 rats) after the last Li-ESWT. These rats were used for checking vascular endothelial growth factor (VEGF) expression either via histological analysis or via reverse-transcriptase polymerase chain reaction (RT-PCR) analysis (28). This was done to assess for early and potentially transient signaling following Li-ESWT. Rats that were used for histological analysis, given that they were treated with EdU at birth, could potentially allow for tracking of progenitor cells as previous reported.

### Application of Li-ESWT and treatment protocol

While conventional shockwave treatment in Urology is performed as a focused high energy pulse for urinary stone disease, Li-ESWT disperses considerably less energy at 0.03–0.06 mJ/mm$^2$, is unfocused, and fewer shocks are administered. Application of Li-ESWT was performed using the MTS Dermagold (Atlanta, GA, USA). The shockwave probe was applied directly to the shaved pelvis of the rat, overlying the area of the urethra and surrounding pelvis, and was coupled to the skin using ultrasound gel with

energy flux density (EFD) of 0.06 mJ/mm$^2$, and 300 shocks at 3 Hz during each session.

### Measurement of LPP

Under anesthesia with urethane, the LPP measurement was conducted as we reported previously (29). In brief, a polyethylene-90 (PE-90) tube (Clay-Adams, Parsippany, NJ, USA) was inserted into the bladder dome. The rat's bladder was slowly filled with warmed phosphate buffered saline, and the bladder capacity was determined by the point at which leakage of urine was noted. This procedure was repeated 3 times and the average bladder capacity was used. The LabView 6.0 software (National Instruments, Austin, TX, USA) was used to record the intravesical pressure. The bladder was filled to 40% of capacity, and increasing manual extravesical pressure was applied until leakage was noted. This procedure was repeated 6 times, and the average pressure at which the rat leaked urine served as the LPP for that particular rat. The rat was subsequently sacrificed, and organs harvested.

### Histological staining

Cryosections of urethral tissue samples were prepared, and Immunohistochemical staining was performed as previously described (25,30,31) using rat endothelial cell antibody (RECA, 1:500, AbD Serotec, Raleigh, NC, USA) to label blood vessels and fluorescence staining of Alexa-488–conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) to label urethral smooth and striated muscular bundles. In the second set of rats receiving EdU injection at birth, sections were assessed for EdU labeling using the Click-iT reaction cocktail (Invitrogen, Carlsbad, CA, USA). The entire urethral section for each rat was surveyed and the number of EdU labeled cells counted. Urethral tissue sections were also prepared for Masson's trichrome staining as we reported previously (1). In brief, the tissue sections were immersed in Bouin solution for 15 min followed by Weigert Hematoxylin staining for 10 min, Biebrich scarlet—acid fuchsin for 3 min, and aniline blue for 3 min.

Images were captured with A Retiga Q Image digital still camera and ACT-1 software (Nikon Instruments Inc., Melville, NY, USA) and analyzed with Image-Pro Plus image software.

### Myodifferentiation of rat myoblast L6 cells in vitro

Rat myoblast L6 cells were used in this experiment and were divided into 4 groups: (I) control; (II) induction; (III) Li-ESWT; and (IV) induction + Li-ESW. Control and Induction groups were cells cultured in DMEM containing 15% FBS and 2% horse serum, respectively. Li-ESW groups were cells treated with Li-ESW at 0.03 mJ/mm$^2$ for 200 shocks. All groups were stained for Myosin heavy chain (MHC) (1:500, Abcam Inc., MA, USA) and Myogenin (1:500, Abcam Inc., MA, USA), followed by microscopy and photography at 7 days.

### Statistical analysis

Data were analyzed with Prism 5 (GraphPad Software, San Diego, CA, USA). One way analysis of variance (ANOVA) followed by the Tukey-Kramer test for post hoc comparisons. The difference was considered significant when $P<0.05$. All data are shown as mean ± standard deviation (SD).

## Results

### Li-ESWT improves urethral LPP

At 5 weeks following VBD, rats underwent functional evaluations with bladder capacity measurement and LPP analysis. Average bladder capacity across groups is shown in *Figure 1A*. The rats that underwent VBD had a decreased average bladder capacity compared to normal controls. In the group of VBD with Li-ESWT, bladder capacity trended towards being greater (improved) than that of the VBD group, though the difference is not statistically significant ($0.6\pm0.10$ versus $0.3\pm0.05$ mL, $P=0.15$).

Average LPPs across groups is shown in *Figure 1B*. The average LPPs for rats receiving VBD was significantly deceased compared to normal controls ($27.0\pm2.9$ versus $51.5\pm6$ cmH$_2$O, $P<0.01$). In the group of Li-ESWT, the average LPPs were significantly improved compared to those of VBD only ($44.8\pm3.2$ versus $27.0\pm2.9$ cmH$_2$O, $P<0.01$). Characteristic LPP curves are shown in *Figure 1C*.

### Li-ESWT increased EdU + progenitor cells in the urethra

To track the endogenous progenitor cells, we modified the label-retaining cell (LRC) method by replacing 5-bromo-2-deoxyuridine (BrdU) with EdU (32,33), which is much easier to detect and the resulting signal is much more reliable (34). EdU was intraperitoneally injected into newborn rats, and VBD was performed when the rats were
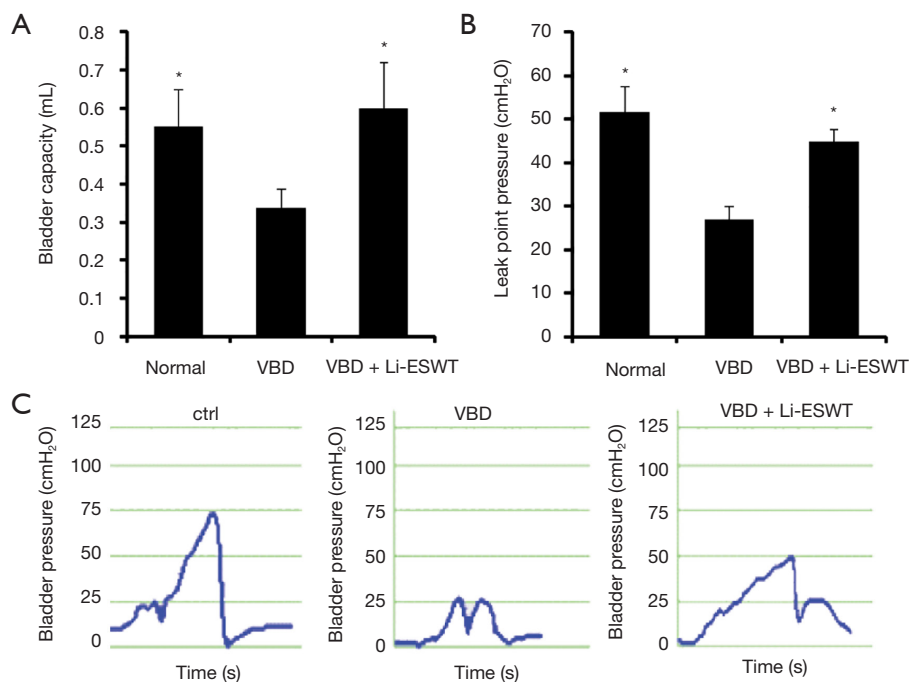
**Figure 1** Functional parameters. (A) Average bladder capacity (mL) (*, P=0.15 compared to VBD); (B) average LPP (cmH₂O) (**, P<0.01 compared to VBD). There is no significant difference of average LPPs between the groups of VBD with Li-ESWT and normal controls (44.8±3.2 versus 51.5±6 cmH₂O, P=0.51); (C) representative LPP curves. VBD, vaginal balloon dilation; LPP, leak-point pressure; Li-ESWT, low-intensity extracorporeal shock wave therapy.

3 months old, Li-ESWT were applied 1 week after VBD. Analysis of the urethras at 24 hours and 1 week after the last Li-ESWT therapy showed significantly increased EdU+ endogenous progenitor cells in the muscle layer, particularly the striated muscle (*Figure 2*).

### Li-ESWT increases urethral angiogenesis by promoting VEGF expression

With Masson's trichrome staining, it was found that rats treated with Li-ESWT after VBD consistently had an increase in venous channels involving all layers of the urethra compared to VBD only and normal control groups (*Figure 3A,B*). Expressions of VEGF in urethral wall were checked with RT-PCR. Compared to the 2 groups of normal control and VBD only, the Li-ESWT treated rats, sacrificed at 24 hours, showed significantly increased expression of VEGF. However, the VEGF expression decreased by 1 week after Li-ESWT. This suggests that in the first 24 hours after Li-ESWT, VEGF is up-regulated, and that this effect diminishes to baseline within 1 week after Li-ESWT (*Figure 3C*). Those results were further

confirmed With Masson's trichrome staining (*Figure 3D*).

### Li-ESWT evokes urethral muscle regeneration in vivo

Urethral staining with phalloidin revealed that the striated muscle layer was attenuated in rats receiving VBD only, appearing thinner and less well organized. These impaired muscle layers showed improvement in the group of Li-ESWT after VBD compared to the VBD only group, characterized by longer and wider myofibers (*Figure 4*) (*P<0.05), suggesting muscle regeneration associated with Li-ESWT.

### Li-ESW promotes myotube formation from rat myoblast L6 in vitro

To further test our hypothesis that Li-ESW can induce muscle regeneration, we checked the effect of Li-ESW on rat myoblast myotube formation in vitro. In medium supplemented with 15% FBS, these L6 cells could be propagated without differentiation and myotube formation. However, in medium containing 2% horse serum (induction
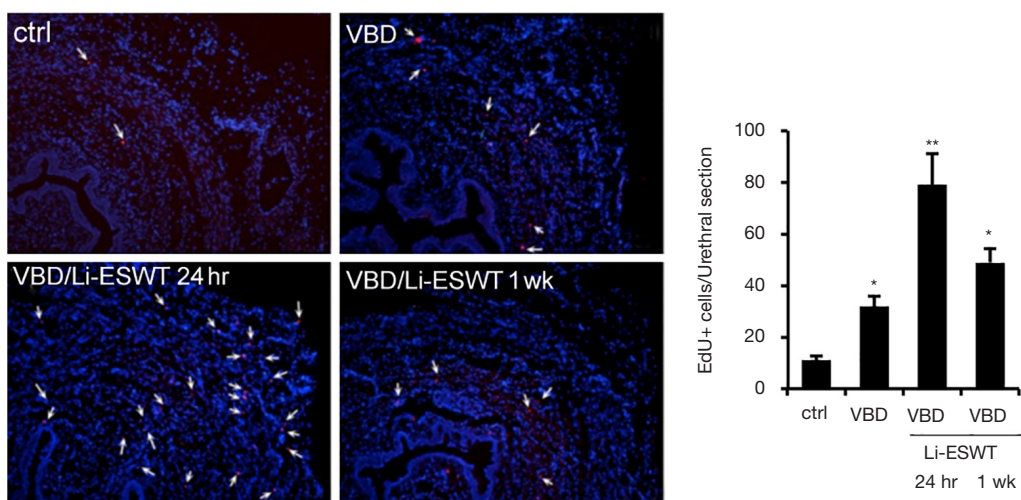
**Figure 2** Li-ESWT increased recruitment of EdU LRCs. Rats with EdU-labeled LRCs were divided into control (ctrl), VBD, and VBD + Li-ESWT. The ctrl, VBD, and VBD + Li-ESWT rats were sacrificed 24 hours and 1 week later. Their urethras were stained for EdU (red) and DAPI (blue) (×100). *, $P<0.05$ *vs.* ctrl; **, $P<0.05$ *vs.* VBD and ctrl. LRC, label-retaining cell; VBD, vaginal balloon dilation; LPP, leak-point pressure; Li-ESWT, low-intensity extracorporeal shock wave therapy; EdU, 5-ethynyl-2-deoxyuridine; DAPI, 4',6-diamidino-2-phenylindole.
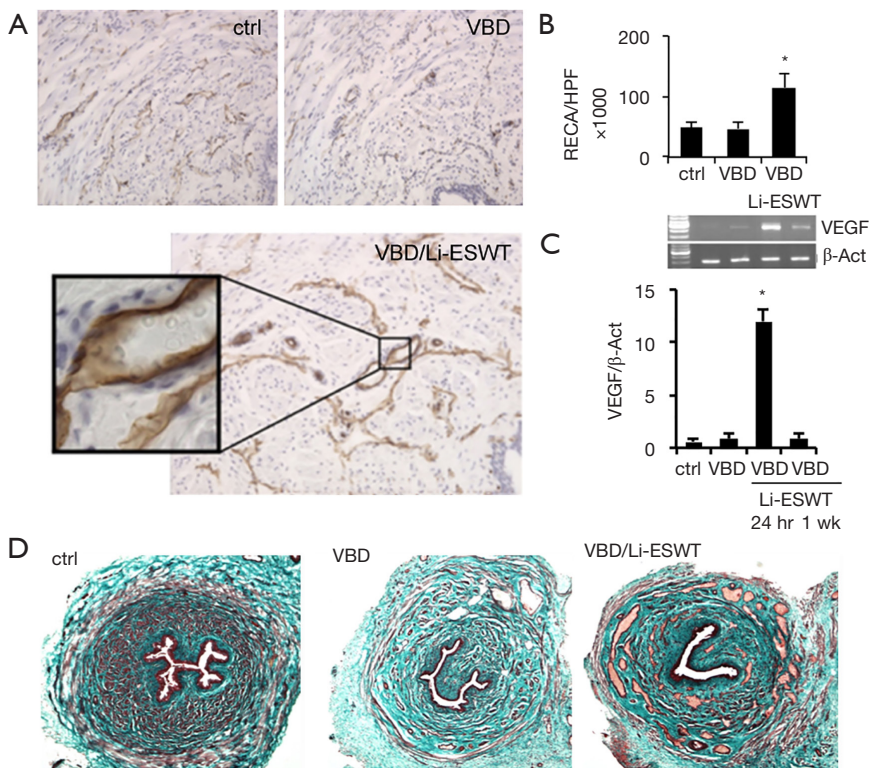


**Figure 3** Urethral angiogenesis affected by Li-ESWT. (A,B) Expression of RECA were significantly increased in the VBD + Li-ESWT group (*, $P<0.01$) (×100); (C) RT-PCR of rat urethras shows increased VEGF expression at 24 hours following Li-ESWT (*, $P<0.01$); (D) increased venous channels seen on trichrome staining in the VBD + Li-ESWT group (×40).
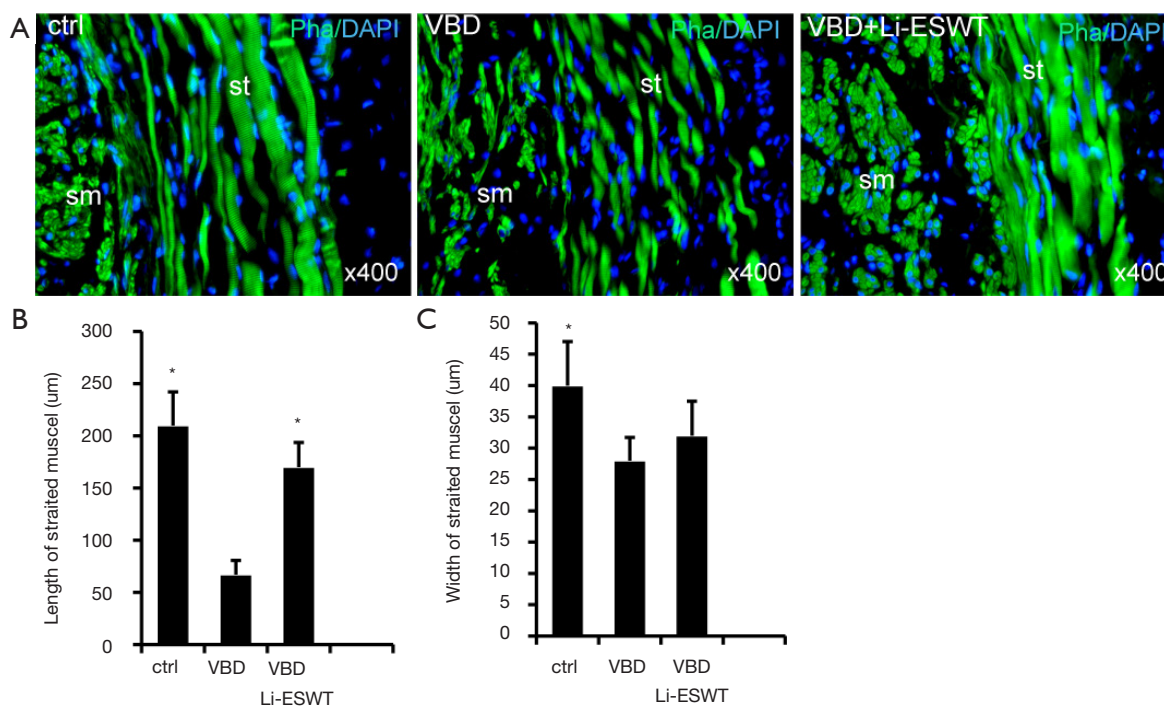
**Figure 4** Li-ESWT promoted urethral sphincter regeneration. The urethral striated muscle fibers (A), as measured by length (B) and width (C) (*, P<0.05 *vs.* VBD). Both smooth (sm) and striated (st) muscles stained green with phalloidin while cell nuclei stained blue with DAPI. VBD, vaginal balloon dilation; Li-ESWT, low-intensity extracorporeal shock wave therapy; DAPI, 4',6-diamidino-2-phenylindole.

medium), the formed myotubes stained positive for MHC and myogenin. Importantly, when treated with Li-ESWT, the induced myotubes grew larger and more robust (*Figure 5*), suggesting satellite cell myogenic differentiation as a possible mechanism for the therapeutic effects of Li-ESWT.

## Discussion

Anatomically, the urethral supportive and sphincteric system are critical to maintain urinary continence (35). Proper sphincteric function is dependent mostly on the urethral striated muscles, but the smooth muscle, mucosa and vascular elements also play a significant role. Urethral sphincter deficiency is the primary culprit in female SUI (36). We and others have confirmed that smooth muscle distributes throughout the entire length of the urethra, while the striated muscle is confined to the proximal half of the structure similar to the urethra in women (1,37,38). As seen in this study and prior study of rat models of SUI after VBD, there is significant diminution in the quality and completeness of urethral striated muscle fibers following VBD (39). Tissue hypoxia may be, in

part, the etiology of this breakdown in striated muscle fibers (40). The findings of increased vascularity in our Li-ESWT treated group may thus lead to a reduction in tissue hypoxia, and may mitigate urethral damage.

In 1998, we established an SUI rat model that mimics closely the female human pathology of SUI (41). This model, best known as VBD, has since been the most widely used SUI model (42). It has been confirmed that in this SUI animal model, both striated and smooth muscles are significantly reduced. Treatment of such models with SCs partially can restore both striated and smooth muscles, but the underlying mechanism remains unknown (8,39,41,43).

In the past decade, despite significant advances in the management of SUI, there are still no therapeutic options capable of reversing the pathophysiologic deficit in the urethral sphincter. The potential of Li-ESWT for the treatment of various urological diseases has been reported in recent years (13,14). As far as we know, this report is the first to investigate the therapeutic effects of Li-ESWT in SUI by using a well-established VBD-induced rat model. Our results showed that Li-ESWT significantly improved LPP in VBD-induced SUI rats. More importantly, we demonstrated that Li-ESWT significantly promoted
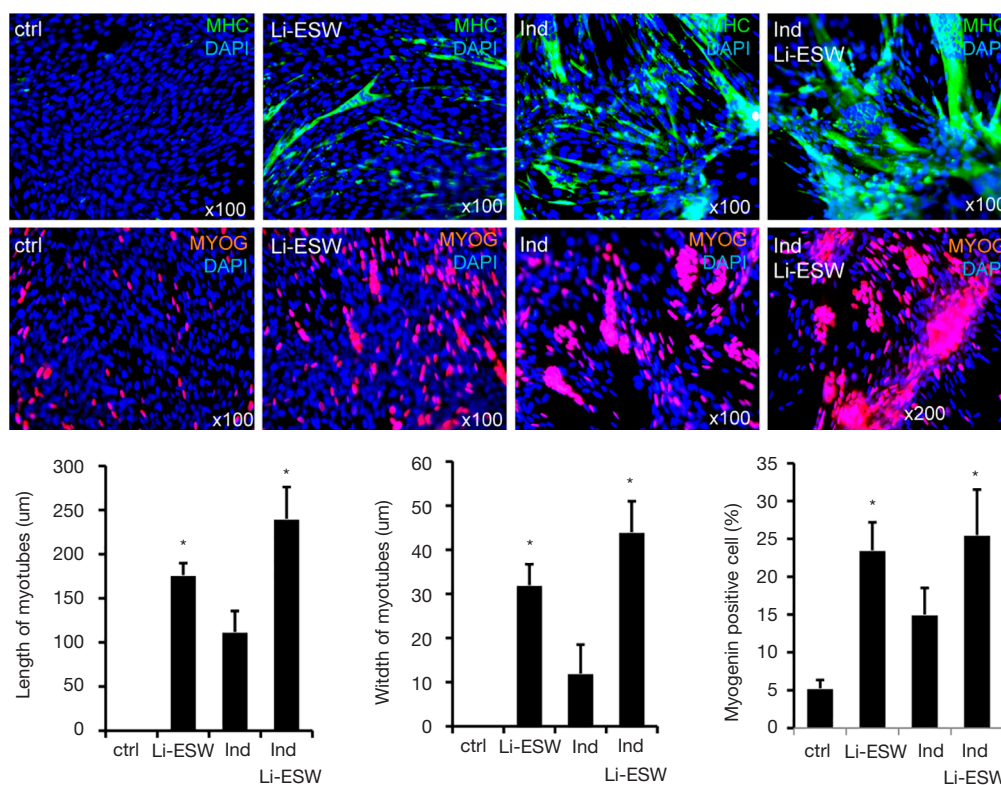
**Figure 5** Li-ESW induces myodifferentiation. Urethral satellite cells were untreated (ctrl), treated with 2% horse serum (Ind), Li-ESW, or Ind + Li-ESW. Seven days later, the cells were stained for MHC (green) and myogenin (red), and the myotubes' length and width determined. *, P<0.05 *vs.* ctrl & Ind. Li-ESW, low-intensity extracorporeal shock wave; MHC, Myosin heavy chain.

angiogenesis and progenitor cell activation in urethra.

Although our functional and histological results demonstrated the therapeutic effects of Li-ESWT in SUI, the mechanism of Li-ESWT in improving urethral function is not understood. As seen in this study and prior study of rat models of SUI via VBD, there is significant diminution in the shape and size of urethral striated muscle fibers following VBD (39). These altered striated muscle fibers appear to have recovered partially following Li-ESWT in the current study. In this VBD induced SUI model, tissue hypoxia has been proposed to be, in part, the cause of breakdown in striated muscle fibers. Multiple prior studies with Li-ESWT in other organ systems have shown benefit through increased angiogenesis (22-24). Our findings of increased vascularity in Li-ESWT treated group might lead to improved tissue oxygenation and thus mitigate urethral damages. Based on these findings, increased vasculature in the urethra may represent one of the therapeutic mechanisms of Li-ESWT in the SUI rat model.

The involvement of progenitor cells in the therapeutic effects of Li-ESWT has been observed in previous studies using rat models of chronic hind limb ischemia and bone defects. Li-ESWT was found to enhance recruitment of endothelial progenitor cells in the ischemic tissue, and also to result in the recruitment of mesenchymal SCs with increased expression of VEGF in the defect tissues. In the current result, we found Li-ESWT also increased the number of progenitor cells in urethra likely due to activation of local progenitor cells as shown in our studies in the penis (17). Our data provide evidence for both theories, promotion of angiogenesis and *in situ* activation of progenitor cells. The former is supported by our finding of increased VEGF expression during the first 24 hours after Li-ESWT, and by our findings of increased vasculature within the Li-ESWT treated rats. The latter is supported by the increase in progenitor cells (labeled with EdU) found in the urethras of the rats sacrificed within 24 hours after Li-ESWT. Thus, it is conceivable that the tissue effects of Li-ESWT on urethra as observed in the present study might have a progenitor cell activation component.

Another significant effect from Li-ESWT on urethra is the regeneration of sphincter, especially urethral striated muscles. This *in vivo* effect was further confirmed by tube formation form rat myoblast L6 cells in vitro. Li-ESWT enhanced the formation of myotube induced by 2% HS in vitro, and, more impressively, these myotubes are wider and stronger compared to induction only. Thus, the underlying mechanism for how Li-ESWT effects SUI is likely related to recovery and repair of urethral sphincteric tissues.

In summary, the present study shows that Li-ESWT was able to significantly restore urethral closure function for in a VBD rat model of SUI. Furthermore, we also showed that these beneficial effects of Li-ESWT were possibly mediated by increased angiogenesis and activation of progenitor cells and sphincter regeneration in urethra. However, this study represents a preliminary study and requires further validation. As such, there are several limitations of this study. First, given that we do not fully understand the mechanism of action or its time course, the Li-ESWT protocol likely requires future adjustment and optimization. Second, the effect of Li-ESWT on the vascular supply to the urethra should be further investigated; and Western blot analysis should be employed to more accurately quantify the endothelial and smooth muscle contents. Third, relatively small numbers of rats, particularly in the set used for EdU and RT-PCR analysis, may limit the significance of our findings.

## Conclusions

Li-ESWT can ameliorate SUI by promoting angiogenesis, progenitor cells activation, and sphincter regeneration of urethra in a rat model induced by VBD, which represents a potential novel non-invasive therapy for SUI in a preclinical setting. To improve our understandings of Li-ESWT therapeutic mechanism and feasibility, more study is needed.

## Acknowledgements

## Footnote

*Conflicts of Interest*: The authors have no conflicts of interest to declare.

*Ethical Statement*: All experimental protocols were approved by the Institutional Animal Care and Use Committee at our institution (approval ID No.: AN109665).

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