Low Intensity Pulsed Ultrasound Influences the Myogenic Differentiation of Muscle Satellite Cells in a Stress Urinary Incontinence Rat Model

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OBJECTIVE	To investigate the therapeutic effect of low intensity pulsed ultrasound (LIPUS) in a stress urinary incontinence (SUI) rat model and its influence on myogenic satellite cells.
METHODS	Fifty Sprague-Dawley rats underwent vaginal distension and bilateral ovariectomy mimicking partum injury and menopause to construct SUI models, which were further randomized into 100 mW/cm^2 LIPUS, 200 mW/cm^2 LIPUS, 300 mW/cm^2 LIPUS, and none-treatment control subgroups with 10 rats per subgroup. Ten rats served as mock operation control. Leak point pressure and bladder capacity were recorded 1 week after LIPUS treatment. Immunofluorescence staining and Western blot were performed to examine histological changes, myodifferentiation, and signaling pathway.
RESULTS	Here, we found the leak point pressure and bladder capacity were restored in 200 mW/cm^2 LIPUS and 300 mW/cm^2 LIPUS groups, but not in 100 mW/cm^2 LIPUS group. More robust striated muscle regeneration was observed in 200 mW/cm^2 LIPUS group comparing with the SUI none-treatment group. Moreover, we found LIPUS activated the myodifferentiation of muscle satellite cells, which is correlated to p38 phosphorylation level.
CONCLUSION	LIPUS restored the leak point pressure and bladder capacity, and activated satellite cell myodifferentiation in SUI rat model. UROLOGY 123: 297.e1−297.e8, 2019. © 2018 Published by Elsevier Inc.

Stress urinary incontinence (SUI) lays substantial social and economic burdens on individuals all over the world. No pharmaceutical treatment for SUI shows reliable long-term efficacy and midurethral slings has a high level of complication rate.[1] Moreover, current surgical treatments fall short of restoring the underlying pathological changes.[2]

Low intensity pulsed ultrasound (LIPUS) is a noninvasive clinical application to promote the healing of bone.[3] It has also been tested in in vivo studies of periodontal wound, acute, and chronic myocardial infarction.[4] Recently, we found LIPUS therapy improved erectile function and reversed pathological changes of penile tissue in STZ-induced diabetic rats.[5] But, there is still little known about the possible effect of LIPUS on SUI model.

Multiple lines of evidence indicated that LIPUS treatment can induce osteoblast differentiation.[6] Other studies demonstrated the regulatory effect of LIPUS on the proliferation and differentiation of several endogenous stem cells including bone marrow stromal cells,[7]

Cardiac mesoangioblasts,[8] mesenchymal stem cells,[9] and osteoblast precursor cells.[10]

Striated urethral sphincter plays a crucial role in urinary continence. After injury, adult striated muscle has a remarkable capability of regeneration in the terms of differentiation and expansion of muscle satellite cells.[11] Muscle satellite cells are endogenous stem cells which are mitotically quiescent and express Pax7 in normal state. When activated by injury, muscle satellite cells begin expressing myoblast determination gene myoblast determination gene (MyoD) and differentiate into myogenic precursor cells, which contribute to muscle regeneration.[12] The role of muscle satellite cells in LIPUS therapeutic effect has yet to be elucidated.

In this study, we investigated the effect of LIPUS treatment in SUI rat model and tried to provide insights into the possible role of satellite cell. Vaginal delivery can lead to injury of nerve, muscle, and connective tissues, which causes continence dysfunction.[13] The vaginal distension followed by bilateral ovariectomy (OV) model in female rats has been

widely used to study the birth−trauma-related SUI.[14] Here, we found LIPUS improved the recuperation of urinary continence and the regeneration of striated urethral sphincter in the VD + OV SUI model. This therapeutic effect depended on muscle satellite cell myogenic differentiation and was related to LIPUS-induced p38 phosphorylation.

MATERIALS AND METHODS

Animal Experiments

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University First Hospital. Fifty eight-week-old primiparous Sprague-Dawley rats were purchased from the Animal Breeding Center of the Peking University Health Science Center. Ten rats were randomly selected as mock operation control group (midline abdominal incision without modeling). Forty rats underwent vaginal balloon dilation followed by bilateral OV to construct SUI models mimicking prolonged labor and menopause as we previously described.[15] The SUI model rats were randomized into none-treatment control group (SUI NC) and 3 LIPUS treatment subgroups (LIPUS 100, 200, and 300) with 10 rats each group. One week after the OV, rats in SUI groups received LIPUS for 3 minutes each time using an adjustable therapeutic machine (WanBeiLi, Beijing, China) with a pulse duration time-to-pulse rest time ratio of 1:4 (200 μs:800 μs) at 1000 Hz and frequency at 1.7 MHz. After hair removal, ultrasonic coupling reagent was applied to cover the abdominal area of the rats. LIPUS was applied through abdomen. The energy intensity applied to SUI NC, LIPUS 100, LIPUS 200, LIPUS 300 groups were 0 mW/cm^2, 100 mW/cm^2, 200 mW/cm^2, and 300 mW/cm^2, respectively. LIPUS treatment was performed every 2 days for 7 times. One week after the last treatment, the leaking point pressure (LPP) and bladder capacity (BC) of all rats were analyzed by cystometry. Rats were sacrificed by bilateral thoracotomy and urethras were harvested for histology and Western blot.

Measurement of Leak Point Pressure and Bladder Capacity

Leak point pressure and BC were recorded 1 week after LIPUS treatment. Rats anesthetized by 10% chloral hydrate (3 mL/kg) received laparotomy to expose the urinary bladder. A polyethylene-90 tube inserted into bladder dome was connected with a saline syringe pump set at 6 mL/hour and a pressure transducer by a T-branch pipe. The voiding pressure curve was recorded by MP150 System (Biopac Systems). Mean BC was determined by 3 cycle of voiding. Mean LPP was determined by 3 times gentle press at bladder by cotton swab to induce a urine leakage.

Masson Trichrome and Immunofluorescence Staining

Five urethra tissues in each group were fixed with 2% formaldehyde and 0.002% picric acid in 0.1 M phosphate buffer for 8 hours, followed by 12 hours of 30% sucrose immersion. Then, the urethra tissues were embedded by optimum cutting temperature compound (Sakura Fine-tek). Cryosections were stained according to standard Masson Trichrome staining protocol. For immunofluorescence analysis, primary antibodies were mouse anti-MHC (Abcam, Cambridge, MA), rabbit anti-Pax7 (Abcam, Cambridge, MA), rabbit anti-MyoD (Abcam, Cambridge, MA), rabbit anti-α-SMA (Abcam, Cambridge, MA). The Alexa Fluor 488 donkey antimouse IgG (Invitrogen, Carlsbad, CA) and Alexa Fluor 594 donkey antirabbit IgG (Invitrogen, Carlsbad, CA) was used as secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). LEICA DFC 425 C digital microscope camera was used to record images. The images were further analyzed by Image-Pro Plus 6.0 software (Media Cybernetics).

Western Blot Analysis

Five urethra tissues in each group were minced and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich). Equal amounts of protein were separated by 10% SDS polyacrylamide gel, electrotransferred to immobilon polyvinylidene difluoride membranes (Merck Millipore) and immunoblotted with antibodies. The primary antibodies used were rabbit anti-p38 (Cell Signaling Technology, Beverly, MA), rabbit anti-phospho-p38 (Cell Signaling Technology, Beverly, MA), mouse anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary antibodies were antimouse and antirabbit IgG conjugated with HRP (Santa Cruz Biotechnologies, Santa Cruz, CA). Membranes were developed for chemiluminescence detection by using ECL detection kit (Pierce) and Universal Hood II image system (Bio-Rad) according to the manufacturer's instruction. All Western blotting analyses were repeated 3 times and analyzed using ImageJ software.

Statistical Analysis

The data are presented as means ± SD. Statistical significance was determined using one-way ANOVA with Student-Newman-Keuls post-test. SPSS 16.0 (SPSS Inc.) was applied for analysis. $P < .05$ was consider to be statistically significant.

RESULTS

LIPUS Improved the Restoration of Urinary Continence in SUI Rat Model

In order to determine whether LIPUS possesses therapeutic benefit to SUI model and screen for an effective parameter, 3 energy intensity (100 mW/cm^2, 200 mW/cm^2, and 300 mW/cm^2) were applied to different

LIPUS treatment groups. Cystometry was performed a week after LIPUS treatment. We found that the LPP in SUI none-treatment control group and 100 mW/cm^2 LIPUS group were significantly decreased (P <.05). In 200 mW/cm^2 and 300 mW/cm^2 groups, LPP were restored to a normal level (Fig. 1A and B). Consistent with these data, the BC was reduced in SUI NC group and 100 mW/cm^2 LIPUS group (P <.05), but restored to a normal level in 200 mW/cm^2 and 300 mW/cm^2 groups (Fig. 1A and C). The 200 mW/cm^2 intensity was used for further study. These results demonstrate that LIPUS improves the urinary continence recovery in SUI model suggesting the therapeutic value of LIPUS in SUI treatment.

LIPUS Promoted Striated Urethral Sphincter Regeneration

To investigate the LIPUS therapeutic effect on the pathological changes of urethral muscles, Masson trichrome staining was performed in mock operation control, SUI NC and 200 mW/cm^2 LIPUS groups. SUI modeling results in a decrease of urethral muscles. The content of urethral muscles was higher in mock operation control and 200 mW/cm^2 LIPUS group than in SUI NC group (Fig. 2A). To better quantify the striated urethral sphincter and smooth muscle respectively, immunofluorescence staining for striated muscle marker MHC and smooth muscle marker α-SMA was performed. The data showed significant improved regeneration of striated muscle
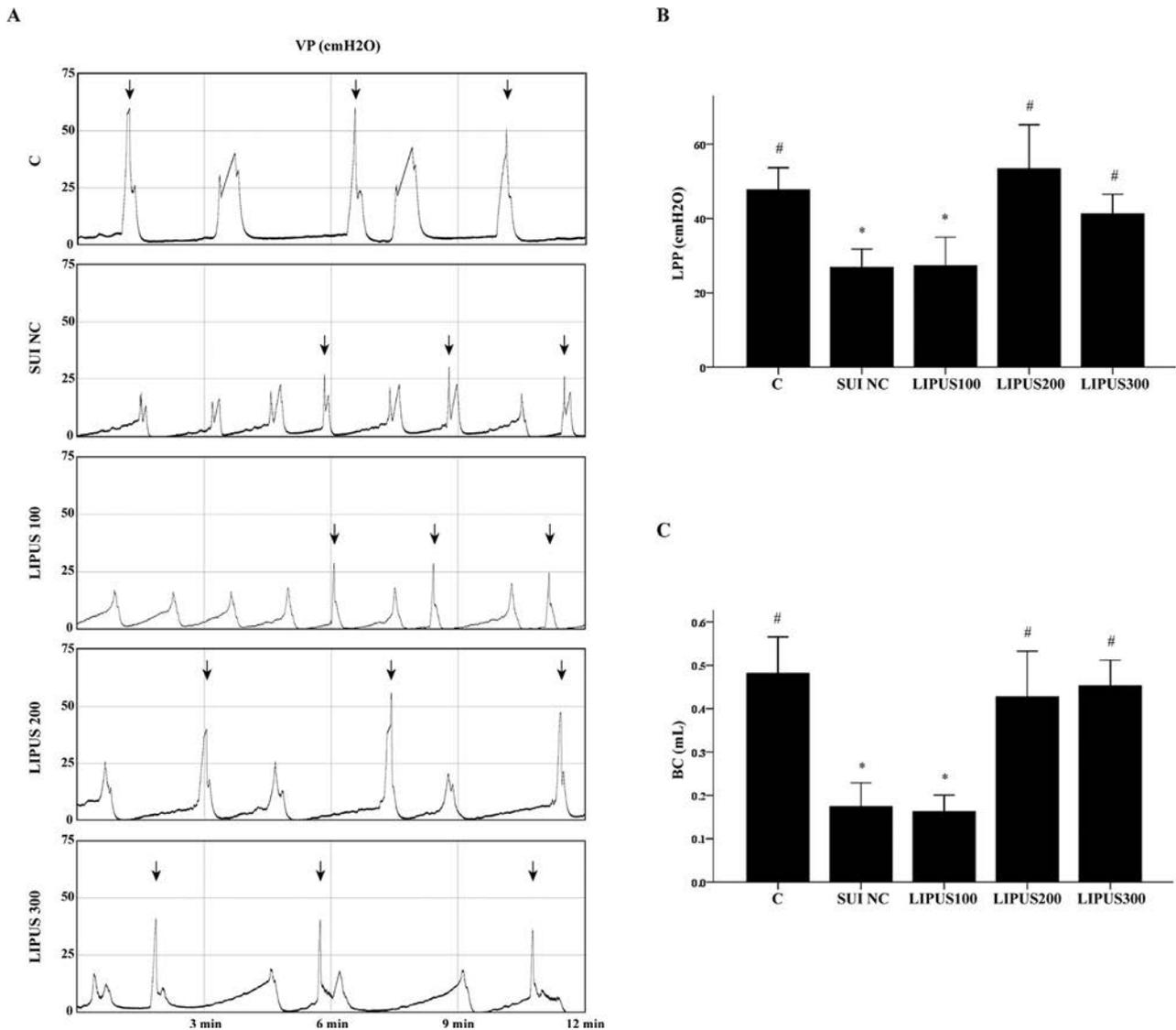

Figure 1. Urinary continence was measured by anesthetized cystometry. (A) Voiding pressure curves were recorded one week after low intensity pulsed ultrasound treatment in LIPUS groups (LIPUS100, LIPUS 200 and LIPUS300), stress urinary incontinence none-treatment control group (SUI NC) and mock operation control group (C). Leak point pressure (arrows) and bladder capacity were recorded. (B) Leak point pressure . (C) Bladder capacity. Three leak point pressure and bladder capacity values were recorded and averaged in each rat. Mean ± SD presented (n = 10 per group). *P <.05 compared with mock operation control group. # P <.05 compared with SUI none-treatment group.

(P $<$.05, Fig. 2B and D), but not smooth muscle (P $>$.05, Fig. 2B and C) in 200 mW/cm^2 LIPUS group. These results indicate that 200 mW/cm^2 LIPUS treatment promote the regeneration of striated muscle that plays an important role in urinary continence function suggesting the pathological mechanism of LIPUS effect.

LIPUS Elevated the Myodifferentiation Level of Muscle Satellite Cells

To explore the mechanism of the improved striated muscle regeneration in LIPUS treatment, the muscle satellite cell marker Pax7 and muscle satellite cell myogenic differentiation marker MyoD were analyzed by immunofluorescence staining. An increased
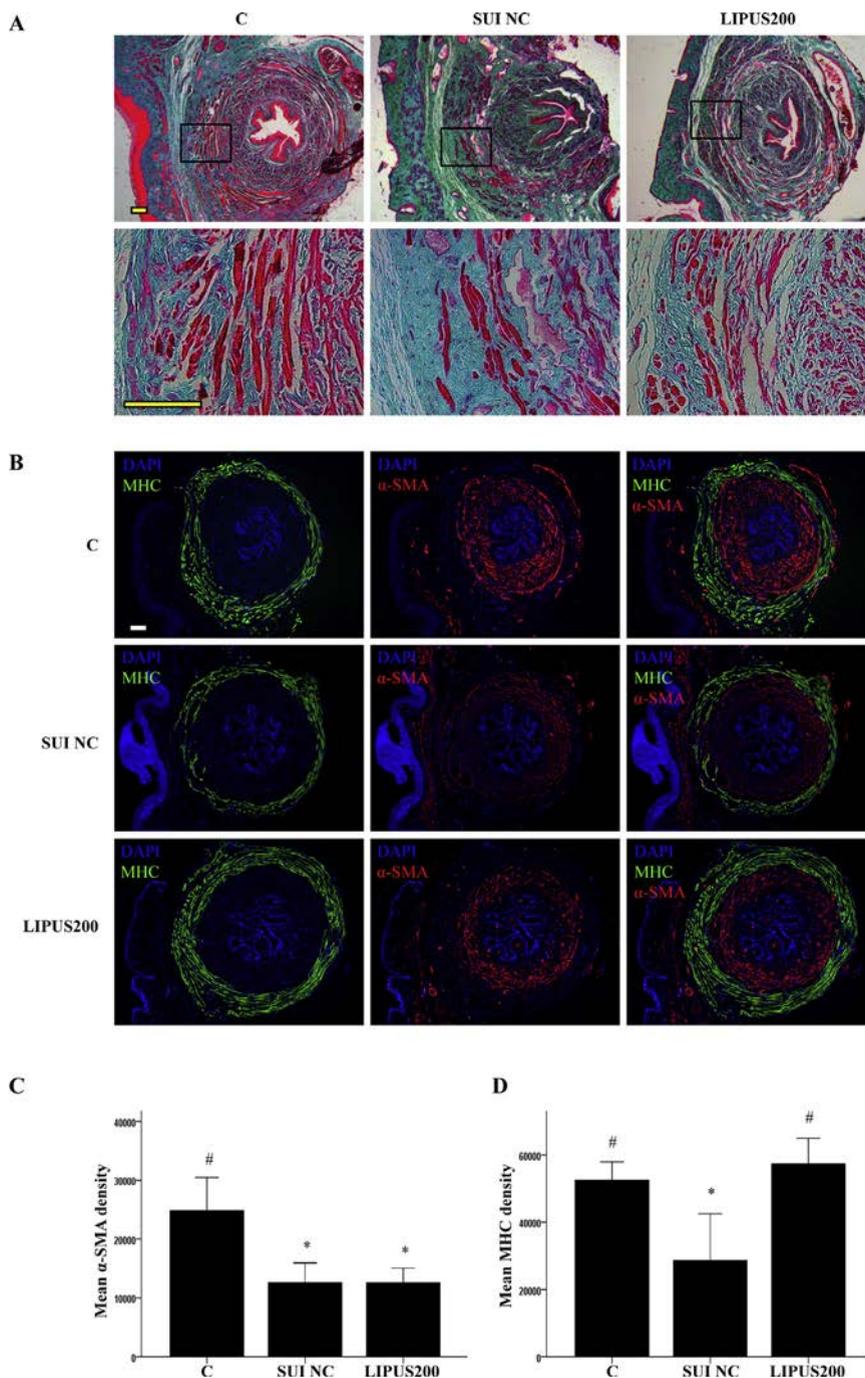

Figure 2. Pathological changes in urethral sphincter. (A) Masson trichrome staining for mock operation control group (C), SUI none-treatment control group (SUI NC) and 200 mW/cm^2 LIPUS group (LIPUS 200). (B) Immunofluorescence staining for striated muscle marker MHC and smooth muscle marker α-SMA. (C) Mean density of α-SMA. (D) Mean density of MHC. Mean $\pm$ SD presented (n=5 per group). *P $<$.05 compared with mock operation control group. # P $<$.05 compared with SUI none-treatment group. Scale bar=20μm. (Color version available online)

number of Pax7$^+$ cells were observed in SUI NC group and LIPUS 200 group ($P < .05$, Fig. 3A and C). No further increase of Pax7$^+$ cells number was found in 200 mW/cm^2 LIPUS group ($P > .05$, Fig. 3 A and C). An elevated level of satellite cell myogenic activation was detected in SUI NC group ($P < .05$, Fig. 3B and D) and no MyoD$^+$ cell was observed in mock operation control group (Fig. 3B and D). In response to LIPUS treatment, myogenic differentiation was more vigorous in 200 mW/cm^2 LIPUS group than in SUI NC group ($P < .05$, Fig. 3B and D). These data indicate the role of the activation of satellite cell myogenic differentiation in LIPUS induced striated muscle regeneration.

LIPUS-induced Myogenic Differentiation was Correlated to p38 Signaling Pathway Activation

P38 mitogen-activated protein kinase (MAPK) signaling pathway plays a role in adult myogenic differentiation triggering the activation of quiescent muscle satellite cells.[16] To further probe into the molecular mechanism of LIPUS-induced myodifferentiation, we analyzed p38 pathway by Western blot. We found the phosphorylation level of p38 was elevated in SUI NC and 100 mW/cm^2 LIPUS groups ($P < .05$), and further elevated in 200 and 300 mW/cm^2 LIPUS groups ($P < .05$, Fig. 4A). These data suggest that LIPUS might regulate muscle satellite cell differentiation through p38 signaling pathway.
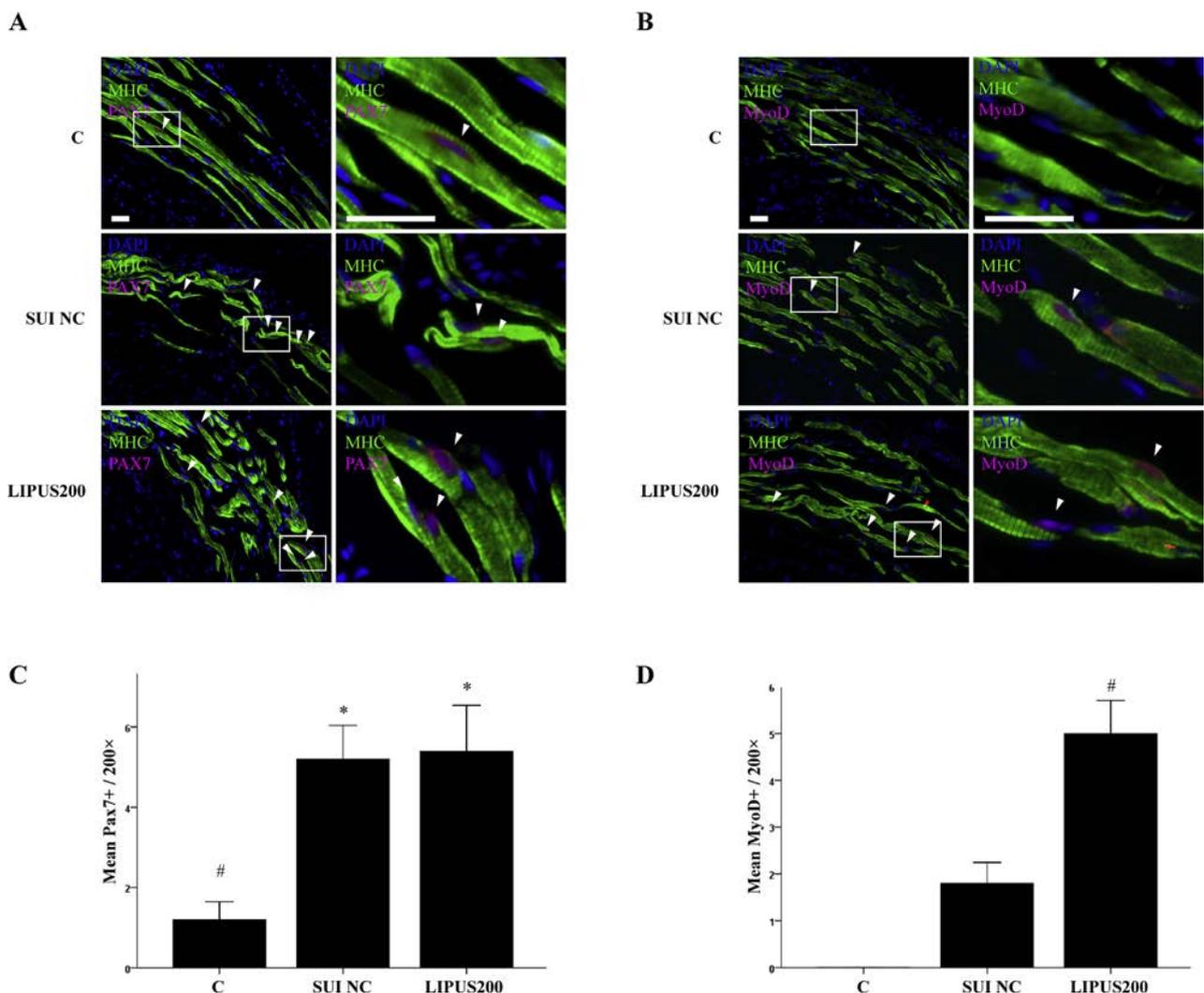

Figure 3. Muscle satellite cell myodifferentiation was activated by LIPUS. (A) Pax7$^+$ cells (arrows) and (B) MyoD$^+$ cells (arrows) in striated urethral sphincter in mock operation control group (C), SUI none-treatment control group (SUI NC) and 200 mW/cm^2 LIPUS group (LIPUS 200). (C) Pax7$^+$ cell count and (D) MyoD$^+$ cell count. Three randomly selected 200 × fields were captured, analyzed and averaged in each rat. Mean ± SD presented (n = 5 per group). *$P < .05$ compared with mock operation control group. # $P < .05$ compared with SUI none-treatment group. Scale bar = 5μm. (Color version available online)
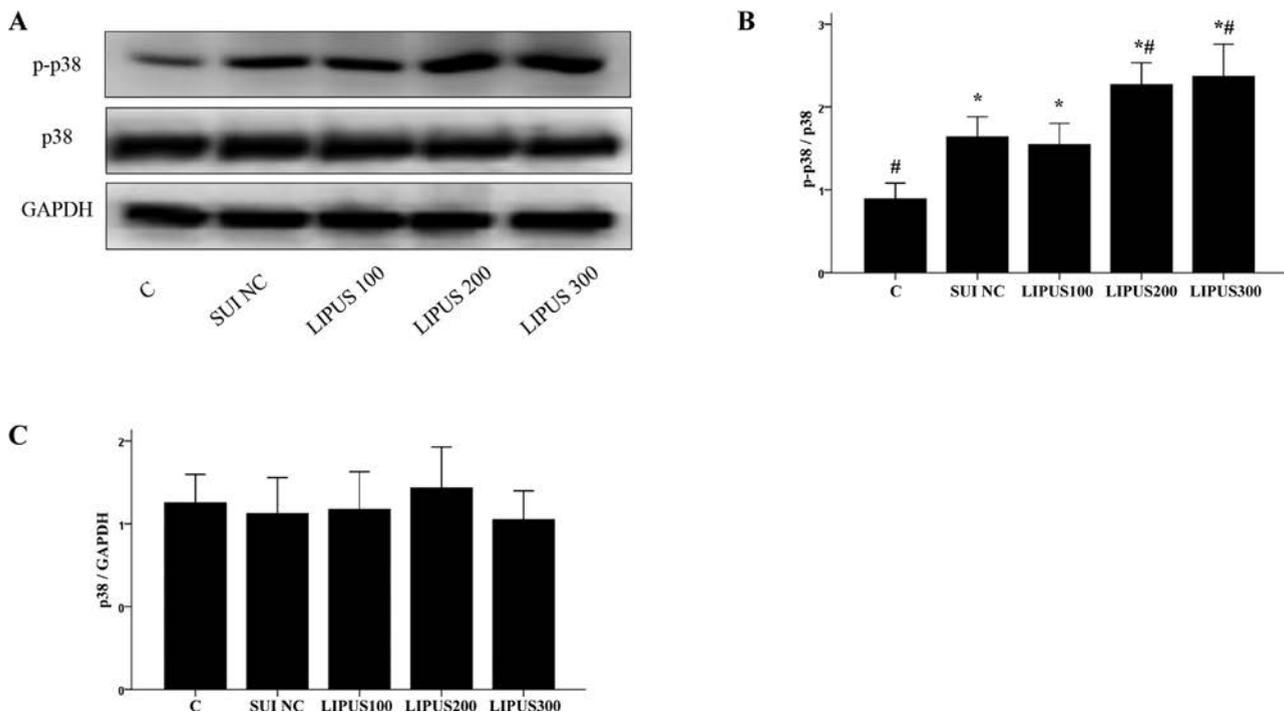
Figure 4. P38 signaling pathway was tested by Western blot. (A) Representative images of Western blot analysis on p38 and p-p38. (B) Quantitative analysis of p-p38 normalized to p38. (C) Quantitative analysis of p38 normalized to GAPDH. Mean ± SD presented (n = 5 per group). *P < .05 compared with mock operation control group. # P < .05 compared with SUI none-treatment group.

DISCUSSION

In our present study, we examined the effect of LIPUS treatment on SUI rat model and observed improved urinary continence after LIPUS treatment. An interesting finding uncovered in this study is the demonstration that LIPUS-induced myogenic differentiation of muscle satellite cell contributes to the striated sphincter muscle regeneration. These results provide experimental evidence for the therapeutic benefit of LIPUS on SUI and the role of muscle satellite cell activation in LIPUS effect.

LIPUS has long been used to enhance and accelerate fracture healing in clinical practices and its effectiveness has been systematically reviewed.[17] Previous study also evaluated LIPUS effect on periodontal tissue healing.[18] The authors found that LIPUS accelerated the soft tissue wound healing as well as bone repair after flap surgery. They suggested that osteoblast and heat shock protein 70 might play a role in the LIPUS effect. Hanawa et al reported that LIPUS ameliorated the left-ventricular ejection fraction in a porcine model of chronic myocardial ischemia via the recovery of capillary density and an upregulation of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS) and basic fibroblast growth factor (bFGF) in the ischemic region.[19] Their group further identified the similar therapeutic effect of LIPUS in an acute myocardial ischemia mouse model.[4] Moreover, we recently reported that LIPUS recovered the

intracavernous pressure as well as the endothelial and smooth muscle content in the penile tissue of a diabetic erectile dysfunction rat model.[5] The observed LIPUS effect was likely to associate with downregulation of the TGF-β1/Smad/CTGF signaling pathway. As a standard surgical treatment for SUI, midurethral slings possess a high risk of complications.[20] Our present study shows LIPUS can restored the (LPP) and BC suggesting that LIPUS might serve as a novel noninvasive strategy for SUI treatment.

SUI is anatomically due to the defects of the urethral sphincter or the support of the urethra. In female urethra, a layer of smooth muscle envelops the full length of the urethra and striated urethral sphincter invests the distal two thirds. Contraction of the striated urethral sphincter closes the urethra, which has crucial contribution to the urinary continence. Recent reports showed that low-intensity extracorporeal shock wave therapy ameliorated SUI by restoring urethral sphincter pathological changes.[21] Our present results demonstrated that LIPUS improved the recuperation of striated urethral sphincter indicating a pathological mechanism of LIPUS effect. The SUI model rats were treated by LIPUS 1 week after injury in our study. Due to the chronic course of SUI in patient population, the therapeutic benefit of LIPUS on SUI patients is largely unclear. The long-term effect of LIPUS on a chronic model merits further investigation. In addition, LIPUS were applied every 2 days for 7 times as a short-term treatment in the present study. Whether

longer period of treatment is capable of maintaining or enhancing its therapeutic effect remains unclear and needs to be further studied.

The influences of LIPUS on the fate of stem cells have been reported in several studies. Naruse et al reported that LIPUS regulated the osteogenic differentiation of osteoblastic cells derived from murine long bone and bone marrow.[22] Wei et al studied the effect of LIPUS on the mesenchymal stem cells (MSCs) migration in bone fracture.[23] They found LIPUS promoted the recruitment of transplanted MSC to the fracture site, which was blocked by the SDF-1/CXCR4 signaling inhibitor. LIPUS-induced cardiac differentiation was reported in mesoangioblasts derived from mouse and human heart.[8] Still, recent report indicated that LIPUS suppressed adipogenesis of MSCs and adipogenic progenitor cell, but promoted osteogenesis of MSCs via kinase-Cot/Tpl2-MEK-ERK signaling pathway.[9] Studies demonstrated that low-intensity extracorporeal shock wave therapy might serve as a noninvasive treatment modality for SUI by promoting progenitor cells recruitment.[21] Our present data demonstrated that LIPUS activate the myogenic differentiation of muscle satellite cells indicating the molecular mechanism of LIPUS-induced striated muscle regeneration. Thus, the mechanical stimulus imposed by LIPUS possesses significant potential to manipulate the differentiation and recruitment of various types of stem cells.

The MAPK cascades have been established as important regulators in stem cell differentiation and proliferation.[24] One of the MAPK pathways, p38 MAPK, participates in adult myogenic differentiation by activating quiescent muscle satellite cells.[16,25] At the early stage of myogenesis, the MyoD/E47 heterodimer transcription complex is induced via p38 pathway following myogenic genes expression.[26] A genetic ablation study of MAPK phosphatase 5 (MKP-5) showed MKP-5 regulate muscle satellite cell proliferation and differentiation via JNK and p38 MAPK pathways respectively.[27] Our previous study has shown that the myogenic and neurogenic differentiation of penile endogenous stem cells can be triggered by icariside II through p38 MAPK pathway.[28] Furthermore, LIPUS has been reported to stimulate the osteogenic differentiation of human periodontal ligament cells through p38 MAPK phosphorylation.[29] Other studies identified ERK pathway as the regulator in LIPUS-induced cellular responses including adipogenesis and osteogenesis.[6,9,30] Our present study showed that LIPUS-induced muscle satellite cell activation was correlated with the phosphorylation level of p38 MAPK. Taken together with our data, LIPUS might influence the fate of different stem cells through distinctive signaling pathways.

CONCLUSION

The present study found that LIPUS-induced satellite cell activation improved the striated sphincter regeneration and restored the (LPP) and BC in SUI rat model, which might mediated by p38 MAPK signaling pathway. These results raise the possibility that LIPUS may serve as a novel noninvasive strategy for the treatment of SUI.

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