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ORIGINAL ARTICLE

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Extracorporeal shock wave therapy decreases COX-2 by inhibiting TLR4-NFκB pathway in a prostatitis rat model

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Abstract

Background: This study aims to evaluate the effect of extracorporeal shock wave therapy (ESWT) on chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) and to explore the mechanism.

Methods: RWPE-2 cells were randomly divided into three groups: (a) RWPE-2 group (normal control), (b) LPS groups (lipopolysaccharide inducing inflammation) and (c) ESWT groups (LPS induced RWPE-2 treated by ESWT). After ESWT was administered, cells and supernatant were collected for enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. In vivo, Sprague-Dawley rats ($n = 30$) were randomly divided into three groups: (a) normal control group, (b) prostatitis groups, and (c) ESWT groups. Prostatitis rats were induced by 17 β-estradiol and dihydrotestosterone for 4 weeks. After ESWT, prostates of each group were collected for immunohistochemistry, Western blot analysis, and ELISA.

Results: ESWT improved prostatitis by attenuating inflammation ($P < .01$). ESWT downregulated the expression of cyclooxygenase 2 (COX-2) through inhibiting TLR4-NFκB pathway compared with the LPS group in vitro or prostatitis group in vivo ($P < .05$). TRAF2 mediates ERK1/2-COX2 pathway. ESWT promotes prostate tissue recovery by stimulating vascular endothelial growth factor expression ($P < .01$). ESWT could suppress apoptosis in the prostate.

Conclusions: ESWT improved CP/CPPS and reduced inflammation by degrading COX-2 in microenvironment through TLR4-NFκB-inhibiting pathway. TRAF2 regulator in ERK1/2-COX-2 inhibition significantly reduced inflammation, thus suggesting ESWT may be a potential and promising treatment for CP/CPPS.

KEYWORDS
chronic pelvic pain syndrome, chronic prostatitis, low-energy shock wave therapy, RWPE-2

1 | INTRODUCTION

Chronic prostatitis (CP) and chronic pelvic pain syndrome (CPPS) are regarded as the most common prostate dysfunctions, which can seriously decrease the life quality of males nowadays.[1] By now the main treatment for CP/CPPS is pharmacological interventions including antibiotics, anti-inflammatory drugs, α-adrenergic blockers, and neuromodulatory drugs,[2-5] but the curative effect is still unsatisfactory.[6] So there is an absolute need for finding an effective and safe approach for CP/CPPS therapy. The major dysfunction symptom of CP/CPPS which disturbs patients is a pain in the prostate area with the absence of any urinary tract infection.[7] Several years

ago, researchers told us that prostaglandin E2 induced by COX-2 contributed to the generation of central sensitization upon peripheral inflammation and inhibited COX-2 expression could relieve pain.[8] In CP/CPPS development, high COX-2 expression also was found and played the main roll in the production of pain.[9] So if there were COX-2 inhibitors or some devices to decrease COX-2 expression, CP/CPPS would be improved. Extracorporeal shock wave therapy (ESWT), as a relatively novel approach in regeneration medicine, has been evaluated as a great potential along with promising evidence, especially for the treatment of various disorders such as tissue trauma and defects.[10] In addition, improvement in injury by ESWT has appeared in a recent report.[11] In our previous study, we found ESWT could effectively improve erectile dysfunction caused by nerve injury and metabolic disorders.[12] Especially in the treatment of nerve injury, ESWT showed a prominent advantage.[13] Some researchers,[14] found that ESWT has reduced inflammatory factors in vivo. Chen et al[15] found that extracorporeal shock wave could decrease ERK1/2 and nuclear factor κB (NF-κB) in vivo. Furthermore, COX-2 expression was proved to be regulated by ERK1/2 and NF-κB.[16] These findings have strengthened the association between ESWT and COX-2.

In this study, we made a hypothesis that ESWT could improve CP/CPPS through decreasing ERK1/2 and NF-κB which can inhibit COX-2 in the prostate. We established a prostatitis rat model and then administered ESWT. We explored the effect of ESWT on CP/CPPS in this experiment and the mechanism behind this approach.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee in the School of Medicine, The Catholic University of Korea (approval number: CUMC-2015-0155-01).

2.2 | Cell culture

RWPE-2 (ATCC, VA) were cultured in low glucose-containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 20% fetal bovine serum (Gibco) and 5 ng/mL basis fibroblast growth factor (Cell Signaling Technology, Danvers) at 37°C at 5% CO_2. Two days later, nonadherent cells were removed and fresh culture medium was added. The culture medium was changed every 2 days. Cells were passaged when they reached approximately 90% confluence.

2.3 | ESWT administration to RWPE-2 cells

RWPE-2 cells were randomly divided into three groups: (a) RWPE-2 group (normal control), (b) LPS group (lipopolysaccharide inducing inflammation), and (c) ESWT group (LPS induced RWPE-2 treated by ESWT). The ESWT was performed by a medical device (Urontech, Hwaseong, Korea) as previously described.[17] Cells in the ESWT group were administered with ESWT treatment, and in normal group or LPS group only sham treatment was performed. The probe was kept in contact with the culture flask containing adherent cells covered with common ultrasound gel. ESWT was performed after cell attachment. Every generation was only treated one time each. Before ESWT and after 12 hours of ESWT, cells and supernatants were collected and stored in −80°C for enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

2.4 | Experimental animal and study design

Fifty-eight-week-old male Sprague-Dawley rats weighing about 270 to 300 g were purchased from a Korean company (Orient Bio Co, Seongnam, Korea). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea. Sprague-Dawley rats ($n = 30$) were randomly divided into three groups: (a) normal control group, (b) prostatitis group, and (c) ESWT group, 10 rats per group. Prostatitis rats were induced by 17 β-estradiol and dihydrotestosterone for 4 weeks. After 1 week, rats in the ESWT group were treated with ESWT and in normal or prostatitis group rats were only treated with sham.

2.5 | ESWT administration to rats

A medical device (Urontech, Hwaseong, Korea) as we previously described was[18] used in this experiment. Under anesthesia, the abdomen of the rat was shaved and exposed in a supine position. Ultrasonic gel was applied to the abdomen, and then a shock wave applicator was placed on the abdomen. A total of 300 shocks were delivered at an energy level of 0.2 mL/mm^2 and a frequency of 120 shocks/min. ESWT was administered once every other day for 4 weeks. After treatment, prostates in each group were collected and stored at −80°C for the next experiment.

2.6 | Immunohistochemistry

The collected cavernous nerve and penis samples were fixed in 4% paraformaldehyde for 24 hours at 4°C before creating a paraffin block. The primary antibodies were used as following: MIP1α (diluted 1:400; Abcam, Cambridge, UK), actin (diluted 1:1000; Abcam), VEGF (diluted 1:500; Abcam), and 6-diamidino-2-phenylindole (Vector Laboratories, Inc, Burlingame, CA) were used to stain the nuclei. Digital images were obtained using a Zeiss LSM 800 Meta confocal microscope (Zeiss, Oberkochen, Germany), and the mean intensity was calculated using ZEN 2012 (Zeiss).

2.7 | Enzyme-linked immunosorbent assay

We quantified the interleukin 6 (IL-6) and IL-8 by species-specific immunoassay ELISA kits (R&D Systems Europe, Abingdon, UK) according to manufacturer's instructions. After ESWT treatment,

cells and tissues were collected and stored at −80°C until the measurement. Absorbance was measured at a wavelength of 450 nm by a microplate reader (Synergy H1 M, Biotek).

2.8 | Western blot analysis

RWPE-2 cells and prostate tissues were homogenized using ice-cold RIPA buffer (Cell Signaling Technology) containing ethylene diamine tetraacetic acid-free protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics GmbH). The homogenized sample was then centrifuged at 12 000*g* for 10 minutes at 4°C and its supernatant was extracted. This supernatant was electrophoresed on NuPAGE 4% to 12% bis-Tris gel (Invitrogen, Carlsbad, CA) and then transferred onto a nitrocellulose membrane. Primary antibodies used include COX-2 (diluted 1:200; Cell Signaling Technology), toll-like receptor 4 (TLR4; diluted 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), NF-κB (diluted 1:400; Cell Signaling Technology), inducible nitric oxide synthase (iNOS; endothelial NOS diluted 1:200; Santa Cruz Biotechnologies), caspase-3 (diluted 1:400; Cell Signaling Technology), TNF receptor-associated factor 2 (TRAF2; diluted 1:200; Abcam), p-TRAF2 (diluted 1:200; Abcam), ERK1/2 (diluted 1:200; Abcam), p-ERK1/2 (diluted 1:200; Abcam), and β-actin (diluted 1:1000; Abcam).

2.9 | Image and statistical analysis

The images were quantified by Image J (Media Cybernetics, Silver Spring, MD). The results were analyzed using SPSS 20.0 software (SPSS Inc, Chicago, IL). The measurement data was presented as mean ± SD and the multigroup comparisons were made with (analysis of variance) followed by the Tukey-Kramer test for posthoc comparisons. Data expressed as proportions were assessed with the χ^2 test. Values of $P < .05$ were indicated as a statistically significant difference.

3 | RESULTS

3.1 | ESWT improved prostatitis by attenuating inflammation

For assessing the inflammation after ESWT, we detected the number of macrophages in the prostate in each group. We found (Figure 1A) after ESWT the number of macrophages decreased, compared with prostatitis group. The quantitative analysis (Figure 1B) showed a consistent result ($P < .01$). Subsequently, inflammation factors like IL-6 and IL-8 were detected by ELISA in vitro and in vivo. The results were shown in Figure 1C and 1D. IL-6 and IL-8 were reduced by ESWT both in vitro and in vivo ($P < .01$). Combination of these results were thought to be improved by

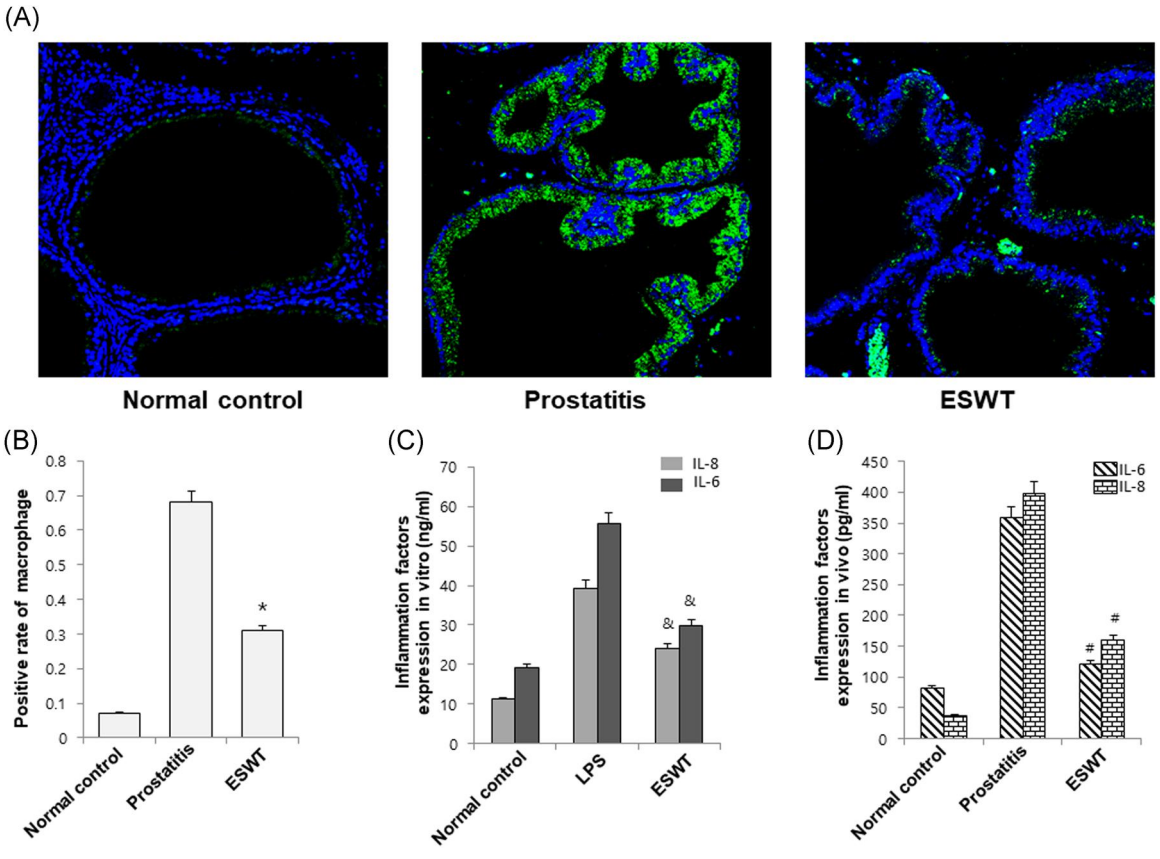

FIGURE 1 A, Representative images of macrophages in prostate for each group. Green is MIP1α, blue is 4′,6-diamidino-2-phenylindole. Original magnification, ×200. B, Quantitative positive rate of macrophages in vivo. $^{*}P < .01$ compared to prostatitis group. C, IL-6 and IL-8 concentration in vitro tested using ELISA. $^{\&}P < .01$ compared to LPS group. D, IL-6 and IL-8 concentration in vivo tested by ELISA. $^{\#}P < .01$ compared to prostatitis group. ELISA, enzyme-linked immunosorbent assay; ESWT, extracorporeal shock wave therapy; IL, interleukin; LPS, lipopolysaccharide; MIP1α, macrophage inflammatory protein 1α [Color figure can be viewed at wileyonlinelibrary.com]

ESWT via decreasing inflammation after ESWT prostatitis in rats. But the mechanism of ESWT to improve prostatitis was still unclear, so we further proceeded into mechanism research.

3.2 | ESWT downregulate expression of COX-2 through inhibiting TLR4-NFκB pathway

Amounts of COX-2 were found in prostatitis or in vitro (Figure 2A and 2a). COX-2 was a main factor of pain generation, so decreasing COX-2 in prostate would relieve the symptom of CP/CPPS (Figure 2B and 2b). In the quantitative analysis of Western blot (Figure 2a and 2b), we found the concentration of COX-2 was downregulated after ESWT. To find the reason, TLR4-NFκB pathway was explored. Figure 2A and 2B illuminated the expression of TLR4 and NF-κB. In comparison with LPS or prostatitis group, the results in the EWST group was higher ($P < .01$), which meant ESWT inhibited TLR4-NFκB pathway. So we held our opinion that ESWT could downregulate the expression of COX-2 by inhibiting TLR4-NFκB pathway in the prostate.

3.3 | TRAF2 mediates ERK1/2-COX-2 pathway

TRAF2 was considered to be associated with the generation of an inflammatory response.[19] In this study, we wanted to find if ESWT will activate the TRAF2. The results of Western blot analysis (Figure 3A and 3B) showed after ESWT more phosphorylated TRAF2 (p-TRAF2) existed in the prostate, which meant ESWT phosphorylated the TRAF2 in rats. And then, p-ERK1/2 and ERK1/2 were tested after ESWT in vivo. Figure 3B displays the quantity result. Our results proved that besides TLR4-NFκB pathway, ERK1/2-COX-2 pathway was also mediated by ESWT in COX-2 generating in the prostate.

3.4 | ESWT promotes RWPE-2 cells recovery by stimulating VEGF expression

Then the ability of RWPE-2 recovery after ESWT was evaluated by testing the VEGF and iNOS. As we can see in Figure 4A, under ESWT the expression of VEGF in the prostate was more. Figure 4B showed the accurate quantitative analysis of VEGF expression, which illuminated that ESWT increased VEGF in RWPE-2 ($P < .01$). In injured tissue, iNOS expression usually upregulated. But in our result (Figure 4C and 4D), we found after ESWT iNOS expression was reduced, which meant ESWT promoted to the recovery of RWPE-2 ($P < .01$).

3.5 | ESWT could suppress apoptosis in the prostate

At last, we assessed the apoptosis in the prostate by testing caspase-3 expression after ESWT. Figure 5A showed the Western blot analysis result of caspase-3 in the prostate for each group. Figure 5B presented the quantity result of Western blot analysis. We found after ESWT the expression of caspase-3 was decreased ($P < .01$), which denoted apoptosis in the prostate was inhibited by ESWT.

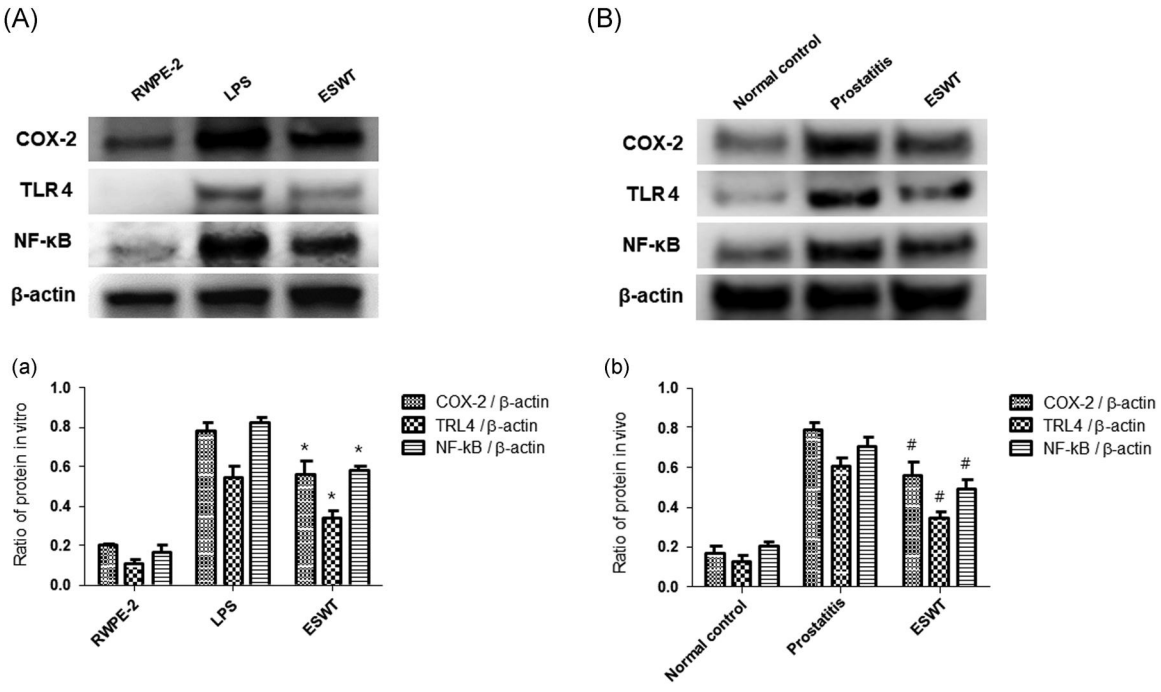

FIGURE 2 ESWT downregulate the expression of COX-2 through inhibiting TLR4-NFκB pathway. A, Western blot analysis results of COX-2, TLR4, and NF-κB in vitro for each group; (a) quantitative analysis of Western blot for COX-2/β-actin, TLR4/β-actin, and NF-κB/β-actin in vitro.*$P < .01$ compared to LPS. B, Western blot analysis results of COX-2, TLR4, and NF-κB in vivo for each group; (b) quantitative analysis of Western blot for COX-2/β-actin, TLR4/β-actin, and NF-κB/β-actin in vivo. #$P < .01$ compared to prostatitis group. COX-2, cyclooxygenase 2; ESWT, extracorporeal shock wave therapy; NF-κB, nuclear factor κB; TLR4, toll-like receptor 4

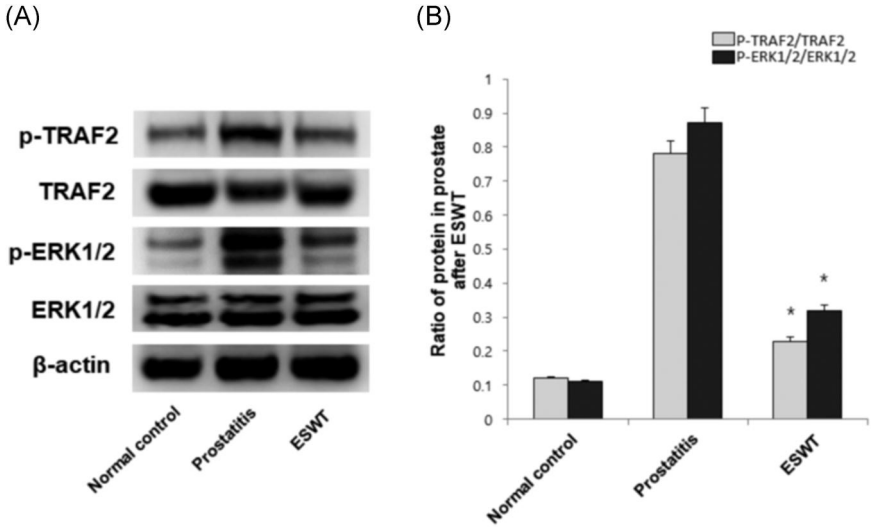

FIGURE 3 TRAF2 Mediates ERK1/2-COX-2 pathway (A) Western blot analysis results of p-TRAF2, TRAF2, p-ERK1/2, ERK1/2, and β-actin in prostate for each group. B, Quantitative analysis of Western blot for-TRAF2/TRAF2 and p-ERK1/2/ERK1/2 for each group. *$P < .01$ compared to prostatitis group. COX-2, cyclooxygenase 2; ESWT, extracorporeal shock wave therapy; TRAF2, TNF receptor-associated factor 2

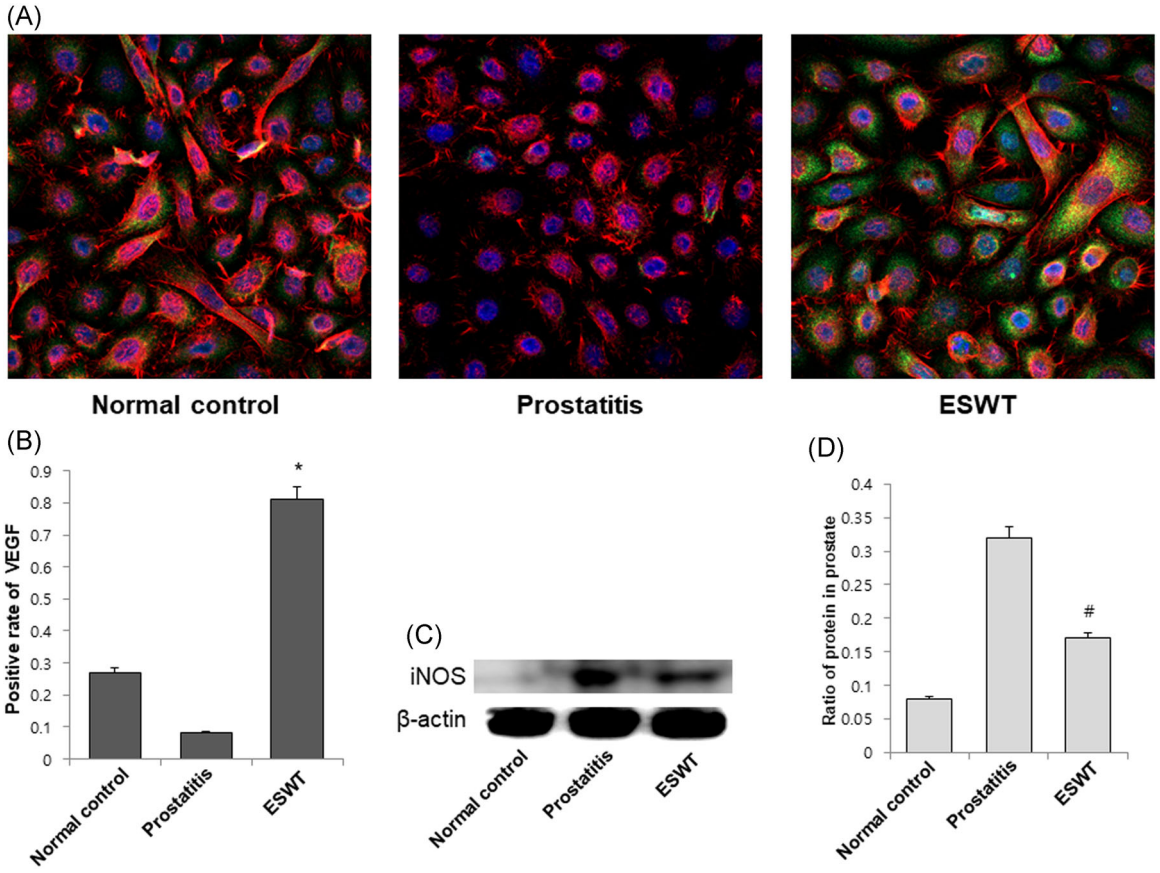

FIGURE 4 A, Representative images of VEGF expression in the prostate for each group. Green is VEGF, red is actin, and blue is 4′,6-diamidino-2-phenylindole. Original magnification, ×400. B, Quantitative positive rate of VEGF in vitro.*$P < .01$ compared to other groups. C, Western blot analysis result of iNOS in vivo for each group. D, Quantitative analysis of iNOS in vivo Western blot for each group. #$P < .01$ compared to prostatitis group. ESWT, extracorporeal shock wave therapy; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Now the unsatisfactory therapy for CP/CPPS is still bothering patients and challenging urologists.[20] In the clinic, the main treatments for CP/CPPS are antibiotics, anti-inflammatory drugs, α-adrenergic blockers, and neuromodulatory drugs.[6] But the mechanism of CP/CPPS is still unclear, which forces us to constantly keep on exploring an effective approach. To search the treatment of CP/CPPS, Chuang et al[21] administered botulinum

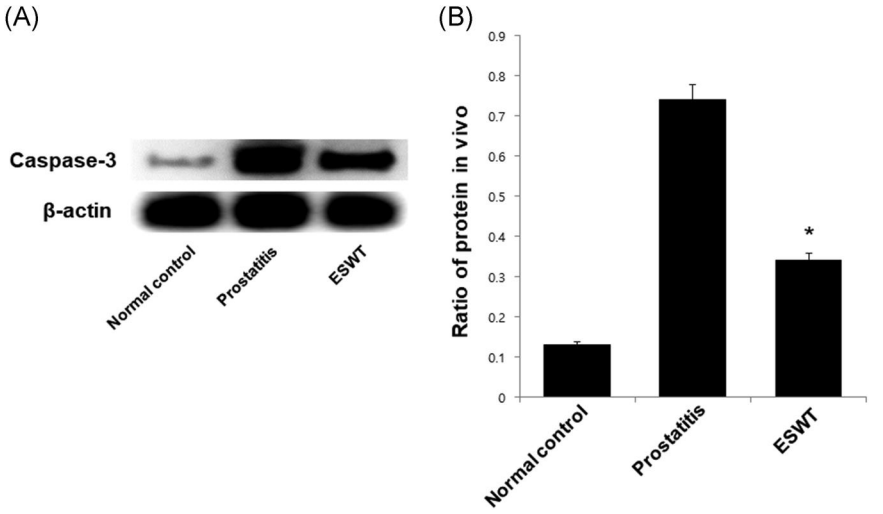

FIGURE 5 A, Representative images of the caspase-3 Western blot analysis in vivo for each group. B, Quantitative analysis of caspase-3 Western blot for each group. *$P < .01$ compared to prostatitis group. ESWT, extracorporeal shock wave therapy

toxin intraprostatic injection in a prostatitis rat model and they found that botulinum toxin found could suppress prostatic pain by inhibiting COX-2 expression. But the reason why prostatitis could induce pain in the prostate region was unexplored. In a previous study, Marszalek et al[22] expounded that pain was the main symptom to the patients with CP/CPPS. This noninflammatory pain symptom was long-term, repeated, and undetermined, which led to treatment difficulty.

In a clinical study, Zimmermann et al[23] had administered the ESWT on patients with CP/CPPS. After a 4-weeks ESWT administration, they collected 12-week follow-ups. Patients after ESWT in their study had an evident improvement. However, the reason why ESWT could improve CP/CPPS was not explored steadily. In our results, we found that after ESWT, inflammatory factors like IL-6 and IL-8 in prostate tissue decreased significantly. And inflammation could be improved with inflammatory factors decreasing in the microenvironment. So this result that ESWT decreased inflammatory factors in prostate tissue also demonstrated that ESWT could alleviate pain which was caused by prostatitis through relieving tissue inflammation and improving neuroinflammation in the prostatitis treatment. Meanwhile, we found that after ESWT, there were more numbers of VEGF and fewer iNOS in the prostate tissue of prostatitis rats. In injured tissue repair, VEGF plays a main role by stimulating cell proliferation.[24] So we thought ESWT could accelerate the prostate tissue repair in a prostatitis rat by stimulating VEGF expression. In injured tissue, iNOS expression was high. As the tissue repair, iNOS expression decreased step by step, which would be normal until repair finish. In this experiment, we found after ESWT iNOS in prostate decreased significantly, which meant ESWT improved the injured prostate. On one hand, ESWT could reduce pain in CP/CPPS by relieving neuroinflammation. On the other hand, ESWT could improve injured tissue repair caused by prostatitis, which reinforced the CP/CPP treatment.

TRAF2 is an important member of the TRAF family, in which TRAF2 was considered to be associated with the generation of an inflammatory response.[18] In a very recent report, researchers[25] found TRAF2 could impact the COX-2, and they noted MAPK and PI3K pathways were involved in this procedure. The MAKP family signaling pathways include three main intermediates (p38, JNK, and ERK).[26] And ERK showed a deep relation with COX-2 expression.[27] So in our experiment, we explored the connection between TRAF2, ERK, and COX-2 under ESWT. And we found a novel phenomenon that as the TRAF2 decreased, which was induced by ESWT, the ERK and COX-2 was also downregulated. This result illuminated that ESWT could mediate the ERK-COX2 pathway via regulating TRAF2 expression. Both TLR4/NF-kB Pathway and ERK1/2-COX2 pathway[25] were inhibited by ESWT, by which COX-2 expression would be decreased in the prostate. The subsequent decrease in COX-2 decelerated the generation of prostaglandin E2, followed by the pain relief of CP/CPPS. Our experiments have some defects indeed. First, for an animal experiment, the ESWT cycle was a little short for prostatitis rats. Because this report is the first experiment about ESWT on prostatitis, which means we had no experience to consult. If the ESWT was administered for a long enough time, we would figure out the optimal treatment program for prostatitis. The reason why we carried out only 1-month treatment was that the prostatitis model would be invalid after a long period of treatment, which could directly interfere with the experimental result. Second, the administration of ESWT was performed at an early stage of prostatitis development, and for the patients lacking the early treatment, the effect of ESWT was hard to make a precise assessment.

5 | CONCLUSION

ESWT improved CP/CPPS and reduced inflammation by degrading COX-2 in microenvironment by inhibiting TLR4-NFκB pathway. TRAF2 regulator in ERK1/2-COX2 inhibition significantly reduced inflammation, thus suggesting ESWT may be a potential and promising treatment for CP/CPPS.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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