



Prostatic fluid exosome-mediated microRNA-155 promotes the pathogenesis of type IIIA chronic prostatitis

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Background: The latest research has shown that exosomes play an important role in cell-to-cell communication and are closely related to the occurrence of many chronic inflammatory diseases. However, no studies have clarified whether exosomes are involved in the pathogenesis of aseptic inflammation, type IIIA chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS-A). This study aimed to explore the relationship between prostatic fluid exosomes and CP/CPPS-A and reveal new pathogenesis.

Methods: Our group collected prostatic fluid samples from CP/CPPS-A patients and normal adult men. Electron microscope, quantitative PCR (qPCR), Western Blot, nanoparticle tracking analysis, hematoxylin-and-eosin (HE) staining, immunofluorescence staining and miRNA-155 functional analysis were used to verify the role of exosomes in CP/CPPS-A *in vivo* and *in vitro*.

Results: Exosomes were abundantly enriched in the prostatic fluid of CP/CPPS-A patients and selectively overloaded with microRNA-155 (*miRNA-155*). These exosomes were taken up by prostatic stromal cells in large quantities. They activated interleukin (IL)-8 and tumor necrosis factor-alpha (TNF- α) expression *in vitro*, and the integrity of the exosomes' plasma membrane is a necessary condition for information transmission by exosomes. In *in vivo* experiments, histological results showed that prostatic fluid exosomes induced prostatitis in rats. Also, immunofluorescence staining showed excessive activation of IL-8, TNF- α , and inducible nitric oxide synthase (iNOS).

Conclusions: Exosomes in the prostatic fluid and the *miRNA-155* contained therein were may be involved with the pathogenesis of CP/CPPS-A.

Keywords: Type IIIA chronic prostatitis; exosomes; microRNA-155 (*miRNA-155*)

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Introduction

Prostatitis is a common disease of the urinary system. Symptoms caused by prostatitis, such as pain, abnormal urination, and sexual dysfunction, seriously affect quality

of life (1). Type III chronic prostatitis, a type of prostatitis known as chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), accounts for approximately 90% of prostatitis cases, with an overall incidence in the population of

approximately 9–15% (2,3). CP/CPPS is a non-bacterial disease and is divided into the more common type-A (non-bacterial inflammatory) and type-B (non-bacterial non-inflammatory) categories according to the white blood cell count in prostatic fluid (4). Because of the particular features of CP/CPPS-A, definitive etiology studies have failed to clarify its pathogenesis. A new entry point is urgently needed in clinical practice to promote further research of CP/CPPS-A.

Exosomes are small vesicles of approximately 30–200 nm in diameter that are generated from cellular endosomes with a double-layer plasma membrane structure and through exocytosis are released by cells into the extracellular space or biological fluids. Exosomes play an important role in cell-to-cell communication (5-7) and are secreted by many cell types, including epithelial and tumor cells (8,9). Exosomes contain abundant lipids, proteins, and microRNAs (miRNAs) from maternal cells and other signaling molecules (10). These substances migrate with exosomes to the recipient cells, thereby regulating the recipient cells' biological states (11). Recent evidence has shown that exosomes are closely related to the occurrence of chronic inflammatory diseases. For example, hepatocytes infected with hepatitis B viruses secreted many exosomes rich in *miRNA-122*. These exosomes were transferred to monocytes to induce the synthesis of interleukin (IL)-1 β and tumor necrosis factor- α (TNF- α). The exosomes secreted by neutrophils deliver *miRNA-155* to vascular endothelial cells to initiate atherosclerosis and promote chronic vascular inflammation (12-15).

MiRNAs are endogenous non-coding RNA molecules that regulate the expression of downstream genes by inhibiting translation or degrading target mRNAs (16,17). They are involved in many physiological processes, such as cell proliferation, cell differentiation, apoptosis, tissue, and organ development, and play a key role in the occurrence and progression of inflammation (18-20). For example, *miRNA-155* promotes inflammatory cytokines and nitric oxide production to regulate the immune response mediated by microglial cells (21); *miRNA-155* is selectively introduced into macrophages to activate TNF- α and IL-6 production, thereby promoting the inflammatory response of lung tissue (22). Importantly, a previous high-throughput sequencing study has shown that *miRNA-155* is significantly increased in the prostatic fluid of patients with chronic prostatitis (23).

Prostate epithelial cells and prostatic stromal cells are two major cell types in prostate tissue and are adjacent

in histological position. A study has shown that prostatic stromal cells are immunogenic, and they secrete cytokines, such as IL-8, under certain stimulating conditions, promote chemotaxis of neutrophils, and regulate the local immune response (24). Also, the prostatic fluid is a special body fluid of great significance to prostatitis diagnosis that is mainly secreted by prostate epithelial cells. However, no reports on the influence of exosomes and their prostatic fluid contents on the occurrence and progression of prostatitis are available. On this basis, this study explored the role of prostatic fluid-derived exosomes and their contained *miRNA-155* in the pathogenesis of type IIIA chronic prostatitis. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tau-21-139>).

Methods

Isolation, identification, and characterization of prostatic fluid exosomes

Collection of prostatic fluid

The seventeen prostatic fluid samples from CP/CPPS-A patients were from patients from the Department of Urology, the First Affiliated Hospital of Army Medical University (Chongqing, China), and Eight volunteers included in the study provided written informed consent before participating in this study healthy adult males were from people randomly recruited from the population. All volunteers included in the study provided written informed consent before participating in this study. The research protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Ethics approval number: KY201801). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Inclusion criteria for patients with CP/CPPS-A were: (I) males between 18 and 55 years old; (II) typical clinical manifestation of chronic prostatitis lasting more than three months; (III) with >10 white blood cells per high-power microscope in routine microscopic examination of prostatic fluid; and (IV) with negative results for common bacteria in urine culture before and after prostate massage. Exclusion criteria for patients with CP/CPPS-A were patients with varicocele, urinary tract infection, urinary tract tumor(s), other diseases in the urinary system, or other systemic diseases. Inclusion criteria for healthy adult males were: (I) 1.18 to 55 years old; (II) without clinical symptoms related

to chronic prostatitis in the past one year; (III) with normal prostatic fluid in routine microscopic examination; and (IV) with negative results of common bacteria in urine culture before and after prostate massage, and the exclusion criteria were individuals having diseases in the urinary system or other systemic diseases.

Isolation of prostatic fluid exosomes

The prostatic fluid sample from each individual was collected in a 1.5-mL sterile Eppendorf tube as described in the literature (25), followed by ultracentrifugation at 3,000 g and 4 °C for 10 min to collect the supernatant and a dilution with an equal volume of sterile phosphate-buffered saline (PBS, pH 7.4). The diluted supernatant was further centrifuged at 10,000 g and 4 °C for 30 min, followed by collecting and filtering the supernatant with a 0.22-mm filter (Millipore, Burlington, MA). The filtered supernatant was further centrifuged at 150,000 g and 4 °C for 2 h in a CP70ME ultracentrifuge (Hitachi, Japan). To further purify the exosomes, the pellet was washed with a large amount of sterile PBS, centrifuged at 150,000 g and 4 °C for 1 h to collect the precipitate, and resuspended in 100 µL sterile PBS for subsequent experiments.

Identification and characterization of prostatic fluid exosomes

A 30 µL exosome sample was placed on a copper mesh and subjected to negative staining at room temperature before observation under an electron microscope (JEOL, Japan). The isolated exosomes were subjected to nanoparticle tracking analysis (NTA) using the NanoSight NS300 instrument (Malvern, UK). Protein markers CD9 and CD63 were used for the characterization of exosomes by Western blot analysis. After collecting the protein samples quantified by Pierce BCA protein assay kit (Thermo Fisher Scientific), the protein samples were mixed with 5×SDS-PAGE loading buffer and incubated at 95 °C for 5 min. After cooling, the sample mixtures were centrifuged at 10,000 g for 1 min to collect the supernatants, which were separated in 15% SDS-PAGE gel (80 V in the concentrated gel for 30 min; 120 V in separating gel for 60 min), followed by transferring the separated proteins to polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes. The protein membranes were blocked in a buffer containing 5% skim milk powder and incubated at room temperature on a shaker for 2 h. The protein membranes were then incubated with primary antibodies (1:1,000 anti-CD9 and 1:1,000 anti-CD63, Thermo Fisher Scientific; 1:1,000 anti-

GAPDH, Santa Cruz, Santa Cruz, CA) independently at 4 °C overnight. After washing in Tris-buffered saline containing 0.5% TWEEN 20 (0.5% TBST solution) three times, the PVDF membranes were incubated with the corresponding secondary antibodies (1:2,000, Cell Signaling Technology, Danvers, MA), which contained 5% skim milk powder at room temperature for 2 h on a shaker. The PVDF membranes were rinsed three times with 0.5% TBST solution (10 min each) and subsequently developed with the enhanced chemiluminescent (ECL) substrate kit (Amersham Biosciences, Piscataway, NJ) before analyzing the results. The GAPDH protein was used as an internal reference control for Western blotting analysis.

The exosomal proteins were quantified by the bicinchoninic acid (BCA) assay, followed by the addition of 10 µL exosome sample with 100 µL RIPA buffer and 1 µL PMSF in a sterile Eppendorf tube, a mixing step, and incubation on ice for 30 min before centrifugation at 12,000 g and 4 °C for 5 min. According to the manufacturer instructions, the collected supernatant was subjected to exosomal protein quantification using a BCA kit (Beyotime Biotechnology, Shanghai, China). The ExoELISA-ULTRA Complete Kit (CD63 detection) (System Biosciences, Palo Alto, CA) was used to detect exosome concentration by adding 20 µL exosome samples with 100 µL coating buffer according to the manufacturer instructions.

Tracing of exosomes and detection of miRNA-155 in exosomes

Cell culture and tracing of exosomes

Normal human prostatic stromal cell line (WPMY-1) purchased from the American Type Culture Collection (Manassas, VA) was placed in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and incubated at 37 °C in an incubator with 5% CO₂, with medium change every 48 h. The cells were sub-cultured when they reached 80–90% confluency.

The exosomes were labeled using PKH26 red fluorescent labeling kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer instructions, followed by co-culturing exosomes with WPMY-1 cells for 12 h and performing DAPI nuclear staining on the WPMY-1 cells. Then, 500 µL DiO cell-labeling solution (Beyotime Biotechnology) was used to label the plasma membrane at 37 °C in the dark for 1 h, followed by rinsing with sterile PBS and observation under a confocal microscope (Olympus, Japan).

Total RNA extraction and quantitative PCR (qPCR) analysis of miRNA-155 concentration

The total RNA of the cells was extracted using TRIzol reagent (Invitrogen) and the Hairpin-it miRNA qPCR Quantitation Kit (GenePharma, Shanghai, China) was used to synthesize cDNA by reverse transcription. Subsequently, the PowerUp SYBR Master Mix (Thermo Fisher Scientific, Waltham, MA) was used for qPCR analysis in the StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA) according to the manufacturer instructions. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal reference for correction, and the target miRNA expression level was calculated using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta C_t}$). The primer sequences used for qPCR were: 5'-ACCCACTCCTCCACCTTTGA-3' for *GAPDH*-forward primer; 5'-CTGTTGCTGTAGCCAAATTCGT-3' for *GAPDH*-reverse primer; 5'-UAAUAGCUAAUCGUGAUAGGGGU-3' for *miRNA-155* primer.

Destruction of exosomal membrane and evaluation of prostatitis *in vitro*

Ultrasound rupture of exosomal membrane

An ultrasonic disruptor (Bioruptor, Diagenode, Liège, Belgium) was used to mechanically disrupt the exosomal membrane structure on ice for 5 min. The cycle period was a 30 s cooling time every 10 s of ultrasonic operation.

Detection of inflammatory cytokines

We tested four groups: blank control (*in vivo*: NS—normal saline; *in vitro*: PBS); normal control (NC—prostatic fluid exosomes of healthy individuals); CP/CPPS-A untreated (CP/CPPS-A—prostatic fluid exosomes of CP/CPPS-A patients); and CP/CPPS-A plus ultrasound-rupture treatment (CP/CPPS-A + US—prostatic fluid exosomes of CP/CPPS-A patients with ultrasound-rupture treatment) groups. In *in vitro* experiments, each exosome group was co-cultured with WPMY-1 cells at 37 °C and 5% CO₂. The supernatants were collected after 48 h, followed by 1,000 rpm for 5 min to isolate the supernatant for detecting IL-8 and TNF- α contents using the corresponding ELISA kits (Absin, Shanghai, China) following the manufacturer instructions.

Evaluation of prostatitis in animal experiments

Experimental animals and prostatitis modeling

Adult male Sprague-Dawley rats weighing 350±20 g were

purchased from the Experimental Animal Center of Army Medical University. The research protocol of animal experiments was reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (Animal ethics lot number: AMUWEC2020939). After routine hair removal and skin disinfection of the lower abdomen and intraperitoneal injection of pentobarbital sodium for anesthesia, each rat underwent lower abdomen incision layer by layer under aseptic surgical conditions to expose the bladder and the prostate behind the bladder. A sterile micro-syringe was used to inject 5 μ L of extracted prostatic fluid-derived exosomes into the left and right ventral lobes of the prostate. The rats in the blank control group were injected with normal saline. Subsequently, the abdomen of each rat was closed layer by layer. All animals were bred routinely after waking up with free access to drinking water, euthanized a week later, and subjected to systemic perfusion and fixation with 4% paraformaldehyde.

Prostate histopathology and immunofluorescence staining

We performed histopathology on rat prostate tissue of different groups. The prostate tissue specimens were paraffin-embedded following the conventional method, followed by sectioning in 5- μ m thick sections and hematoxylin-and-eosin (HE) staining. In the immunofluorescence staining, the sections were thoroughly washed with 0.01 mol/L PBS and incubated in deionized water containing 3% H₂O₂ for 10 min to block the endogenous peroxidase, followed by incubation with rabbit anti-IL-8 (1:200, Affinity Biosciences, Cincinnati, OH, USA), rabbit anti-iNOS (1:200 Affinity Biosciences), or goat anti-TNF- α (1:200, R&D Systems, Minneapolis, MN) at 4 °C overnight. After washing steps, the tissue sections were incubated with the corresponding secondary antibodies [TRITC goat anti-rabbit immunoglobulin G (1:100, Abcam) and FITC rabbit anti-goat immunoglobulin G (1:100, Abcam)] at room temperature for 1 h, followed by DAPI nuclear staining for 10 min, mounting the slides, and observation under a fluorescence microscope (Zeiss, Germany).

miRNA-155 functional analysis

A day before cell transfection, the WPMY-1 cells were inoculated into a 24-well plate containing 500 μ L culture medium until reaching 50–80% confluency.

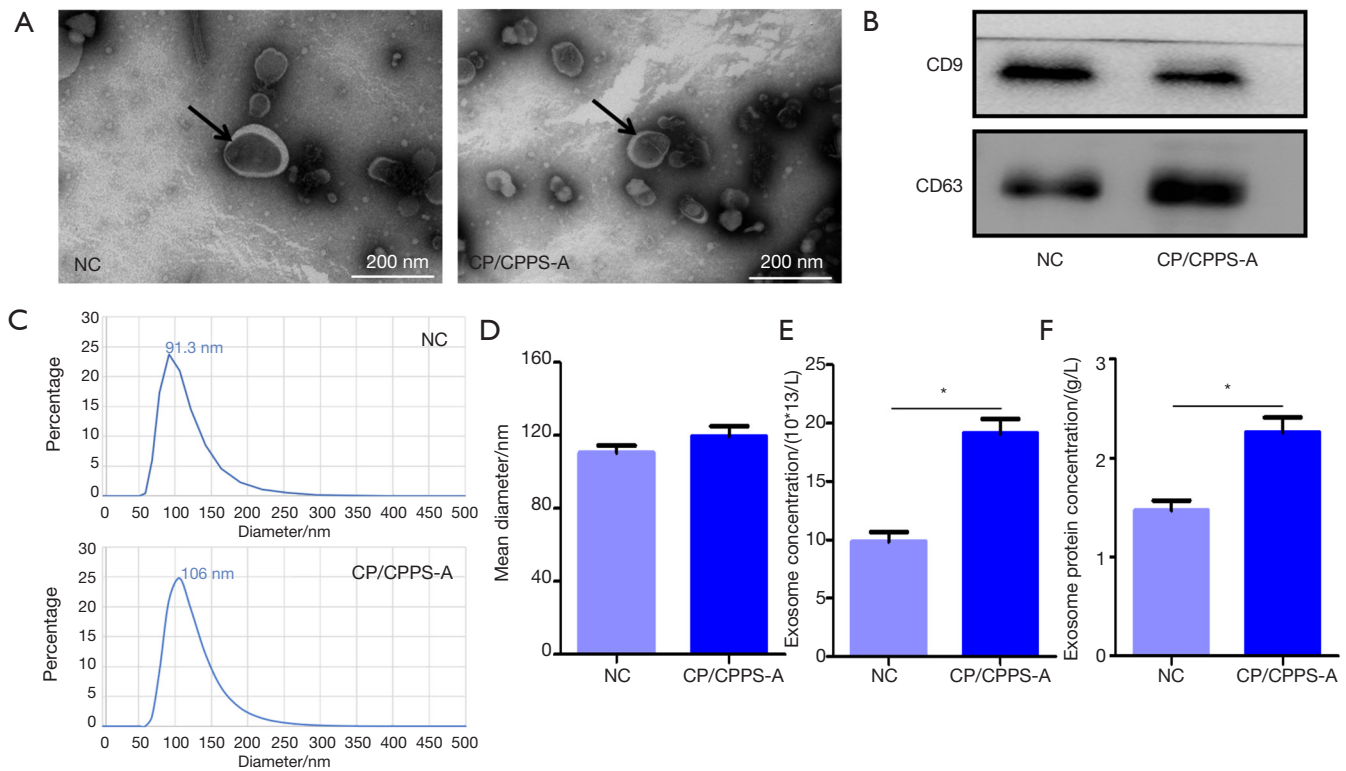


Figure 1 Isolation, identification, and characterization of prostatic fluid exosomes. (A) Electron transmission microscopy observed the prostatic fluid exosomes from a healthy individual and a CP/CPPS-A patient. Scale bar = 200 nm. (B) Western blotting analysis of exosomal surface protein markers, CD9, and CD63. (C) Exosomal diameter distribution is shown by NTA. (D) The average size of exosomes derived from NTA. (E) Results of the concentration of exosomes in prostatic fluid. (F) Exosomal protein concentration measured by the BCA method. *, $P < 0.05$. NC, normal control; CP/CPPS-A, type IIIA chronic prostatitis/chronic pelvic pain syndrome.

Lipofectamine 2000 (Invitrogen) reagent was used according to the manufacturer's instructions to transfect the *miRNA-155* mimics/inhibitor and NC *miRNA-155* mimics/inhibitor (mirVana™, Invitrogen) into the WPMY-1, with mimics of 50 nM and inhibitors of 100 nM in both. The effects were tested 24 h after transfection using qPCR and ELISA described as aforementioned.

Statistical analysis

Quantitative data were presented as a means \pm standard error of the mean (SEM). Comparisons of means were performed using Student's *t*-test or one-way ANOVA followed by Dunnett's post hoc test using SPSS 23.0 software (IBM SPSS, Armonk, NY). The threshold of statistical significance was set at $P < 0.05$.

Results

Isolation, identification, and characterization of human prostatic fluid exosomes

This study successfully isolated exosomes from human prostatic fluid by ultracentrifugation and observed them by electron microscopy. Transmission electron microscopy showed that exosomal vesicles had a typical double-layer plasma membrane structure, elliptical or circular, with a diameter of approximately 40–150 nm (Figure 1A). Western blotting analysis for detecting surface protein markers of exosomes showed positive CD9 and CD63 expression (Figure 1B), which further proved the successful isolation of exosomes in this study.

To further study the characteristics of the isolated exosomes, NTA showed no significant difference in the peak or diameters of prostatic fluid exosomes between

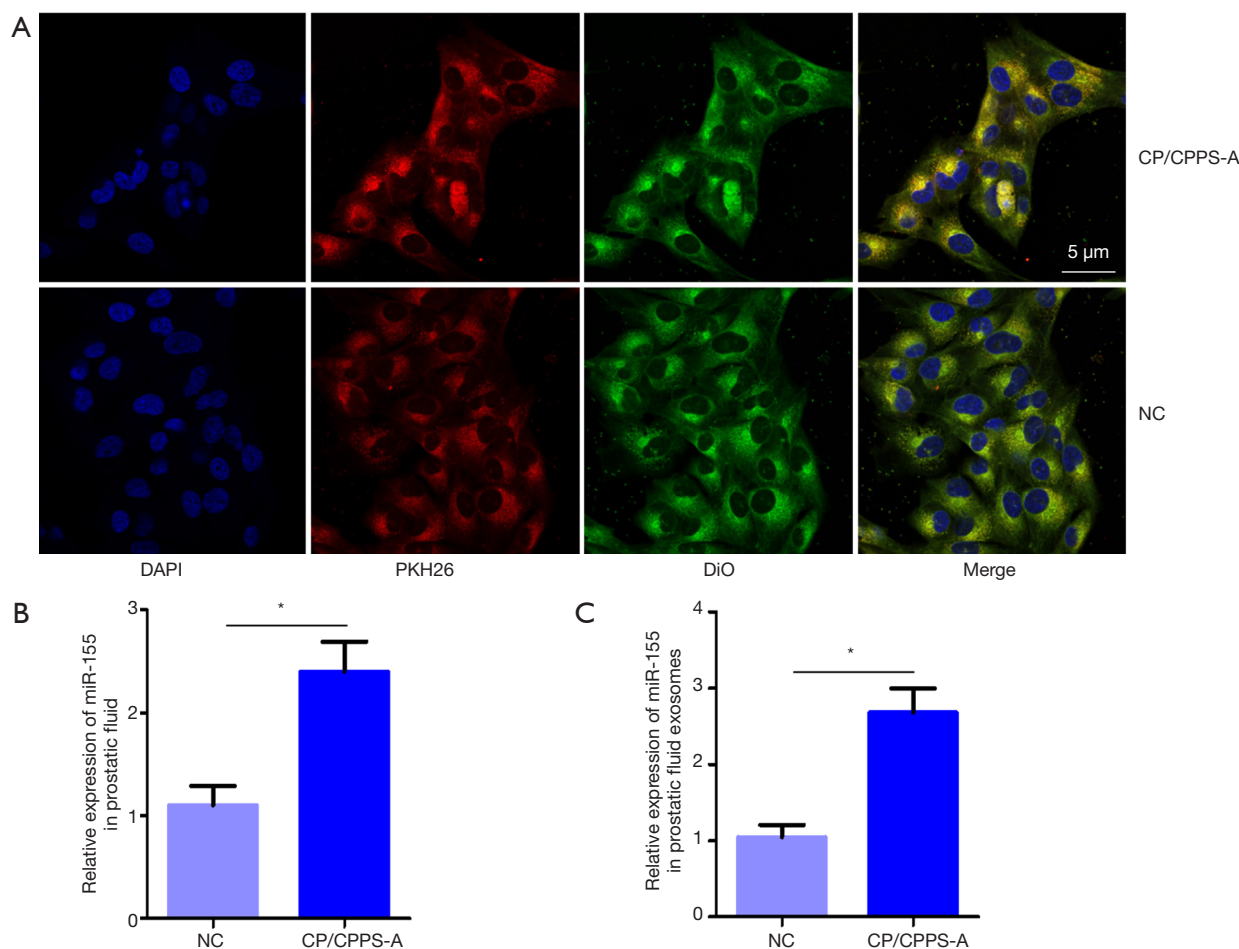


Figure 2 Exosomes were selectively loaded with *miRNA-155* and taken up by prostatic stromal cells. (A) The prostatic fluid exosomes of the healthy individual and CP/CPPS-A patient marked by PKH26 were significantly engulfed and taken up by the prostatic stromal cells. (B) The relative *miRNA-155* expression in NC's prostatic fluid and the CP/CPPS-A groups measured by qPCR. (C) The relative *miRNA-155* expression in prostatic fluid exosomes of the NC and the CP/CPPS-A groups measured by qPCR. *, $P < 0.05$. NC, normal control; CP/CPPS-A, type IIIA chronic prostatitis/chronic pelvic pain syndrome.

healthy individuals and CP/CPPS-A patients (Figure 1C,D). However, the detection of exosome concentrations showed that the exosome concentration in CP/CPPS-A patients was significantly higher than that of healthy individuals (Figure 1E). Similarly, the protein concentrations of exosomes in the CP/CPPS-A patients were also significantly higher than those in healthy individuals (Figure 1F), suggesting that exosomes were excessively secreted in the prostatic fluid of the CP/CPPS-A patients.

Prostatic fluid exosomes transport *miRNA-155* to the prostatic stromal cells

To clarify whether exosomes in prostatic fluid were taken

up by the WPMY-1 cells, we used PKH26 dye to label the exosomes in red and then co-cultured them with WPMY-1 human prostatic stromal cells, followed by the addition of DiO and DAPI to label the cell membranes and nuclei, respectively. Confocal microscopy showed that exosomes successfully entered the WPMY-1 cells, confirming that prostatic fluid exosomes were engulfed by the prostatic stromal cells in large amounts (Figure 2A).

We also tested the concentrations of *miRNA-155* in prostatic fluid and exosomes in different groups. Our results showed that the concentrations of *miRNA-155* in the prostatic fluid and exosomes of CP/CPPS-A patients (CP/CPPS-A group) were significantly higher than in the healthy individuals (NS group) (Figure 2B and 2C),

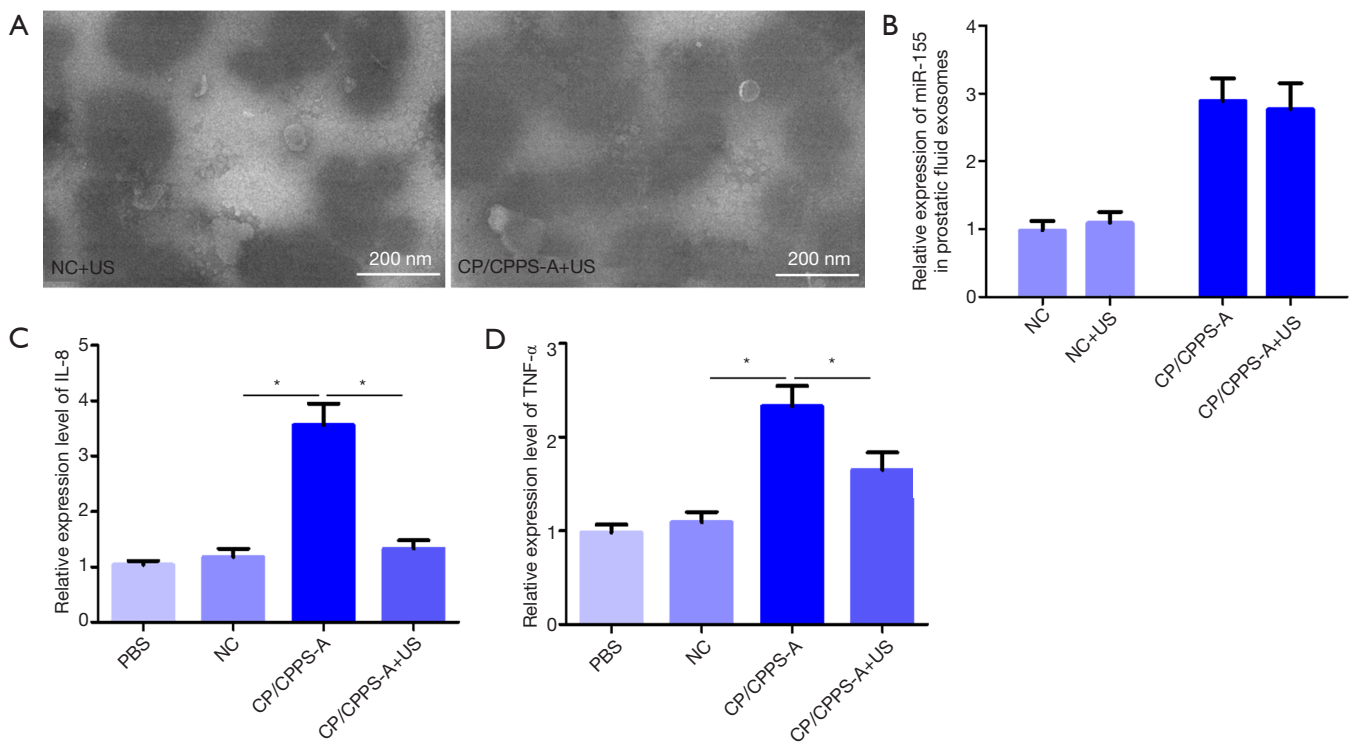


Figure 3 Prostatic fluid exosomes induced inflammation of prostatic stromal cells. (A) Representative images of transmission electron microscopy of prostatic fluid exosomes after ultrasound-rupture treatment extracted from a healthy individual and a CP/CPPS-A patient, scale bar = 200 nm. (B) Relative miRNA-155 expression in the prostatic fluid exosomes of healthy individuals and CP/CPPS-A patients before and after the ultrasound-rupture treatment. (C,D) Relative IL-8 and TNF- α expression levels of exosomes co-cultured with prostatic stromal cells co-cultured in different groups detected by ELISA. *, $P < 0.05$. PBS, phosphate buffer saline; NC, normal control; CP/CPPS-A, type IIIA chronic prostatitis/chronic pelvic pain syndrome; CP/CPPS-A + US, ultrasonic treatment in the CP/CPPS-A.

suggesting that *miRNA-155* was selectively loaded into exosomes of CP/CPPS-A patients and was taken up by prostatic stromal cells.

Prostatic fluid exosomes induce inflammation of prostatic stromal cells

We performed ultrasonic treatment on prostatic fluid exosomes to destroy the exosomes' double-layer plasma membrane structure, followed by observation of the effect of ultrasonic treatment using a transmission electron microscope. Our results showed that after ultrasonic treatment, the intact exosome plasma membrane structure was rarely observed in both NC plus ultrasound-rupture treatment (NC + US—prostatic fluid exosomes of healthy individuals with ultrasound-rupture treatment) and CP/CPPS-A + US groups by electron microscopy (Figure 3A), indicating that most of the exosome membranes were

destroyed. Second, we retested the concentration of *miRNA-155* in exosomes after ultrasonic treatment and showed that the *miRNA-155* concentration did not change significantly with ultrasonic destruction of the exosomal membrane (NC vs. NC + US and CP/CPPS-A vs. CP/CPPS-A + US, Figure 3B).

To observe prostatic fluid exosomes' effect on prostatic stromal cells, we co-cultured the WPMY-1 cells with PBS and with different groups of exosomes (i.e., NC, CP/CPPS-A, and CP/CPPS-A + US groups). After 48 h, we detected IL-8 and TNF- α concentrations using ELISA. Our results showed that IL-8 and TNF- α concentrations of the CP/CPPS-A group were significantly higher than those of the NC group. Also, the IL-8 and TNF- α concentrations of exosomes with ultrasonic treatment in the CP/CPPS-A (CP/CPPS-A + US) group were significantly lower than those of the exosomes without ultrasonic treatment in the CP/CPPS-A group (Figure 3C and 3D). These results

indicated that exosomes derived from prostatic fluid in the CP/CPPS-A patients induced inflammation, which was greatly weakened by ultrasonic treatment.

Prostatic fluid exosomes promote inflammation of the prostate tissue

In the animal experiments, we injected the same amount of normal saline or corresponding exosomes into the ventral lobe of the prostate of male SD rats in individual groups (i.e., NS, NC, CP/CPPS-A, and CP/CPPS-A + US). The animals were euthanized a week after to collect the prostate tissue for paraffin-embedding and sectioning. HE-staining results showed that sections of the exosomes injected with normal saline and normal control prostatic fluid had normal prostate morphology, intact acinar structures, and no obvious inflammatory-cell infiltration in the interstitial tissues. However, prostatic fluid exosomes and sections of prostatic fluid exosomes of the CP/CPPS-A patients treated with ultrasound had many inflammatory-cell infiltrations in the interstitial tissues. Importantly, the inflammatory-cell infiltration in the ultrasonic treatment group was significantly improved compared with the untreated group (Figure 4A).

To further investigate the prostate tissue's inflammation, we used immunofluorescence staining to detect the expression of IL-8, TNF- α , and iNOS in the prostate tissue. Our results showed that the above cytokines were not significantly expressed in the NS and NC groups but were very significantly expressed in the CP/CPPS-A group. Compared with the NC group, the CP/CPPS-A + US group had a higher expression of IL-8, TNF- α , and iNOS, which was significantly lower than in the CP/CPPS-A group (Figure 4B,C,D).

miRNA-155 promoted inflammation of prostatic stromal cells

Considering the prostatitis effect of exosomes derived from prostatic fluid in patients with CP/CPPS-A and the significant upregulation of *miRNA-155*, we verified whether *miRNA-155* could mimic the inflammatory response induced by exosomes using commercially available *miRNA-155* inhibitor or mimic and negative control inhibitor or mimic to perform miRNA functional analysis. After transfection of prostatic stromal cells, the results showed that 50 nM *miRNA-155* mimic significantly enhanced the expression of *miRNA-155*, and 100 nM

miRNA-155 inhibitor significantly reduced the expression of *miRNA-155* (Figure 5A).

We used ELISA to detect the various inflammatory cytokines in the transfected prostatic stromal cells. The enhanced expression of *miRNA-155* increased IL-8 and TNF- α expression. Also, IL-8 and TNF- α expression did not change significantly after inhibiting *miRNA-155* expression (Figure 5B,C), suggesting that high *miRNA-155* expression promoted inflammation prostatic stromal cells, whereas low *miRNA-155* expression did not have an inflammatory protective effect.

Discussion

Although many studies have been conducted on prostatitis, traditional research ideas on inflammation have not clarified the pathogenic mechanisms of prostatitis. Increasing evidence has shown that the main role of exosomes is not as a carrier for cell excretion but rather as a key signal carrier in the process of short- and long-distance cell signaling (26). As mentioned in the introduction, exosomes are secreted into various body fluids, including prostatic fluid and urine. Many studies have suggested that exosomes correlate strongly with certain chronic inflammatory diseases (12-15). We speculate that exosomes and their carried contents may play an important role in the occurrence and progression of CP/CPPS-A.

In this study, we successfully extracted and identified exosomes from human prostatic fluid and found a significant difference in the concentrations of prostatic fluid exosomes between CP/CPPS-A patients and healthy individuals. Histologically, prostate epithelial cells and prostatic stromal cells are adjacent to each other, creating conditions for exosomes to pass between these two types of cells. Also, many researchers believe that prostatic stromal cells are immunogenic in their studies (24). To further verify whether prostatic fluid exosomes were involved in initiating prostatitis, we used the PKH26 dye to label the prostatic fluid-derived exosomes and co-cultured labeled exosomes with human prostatic stromal cells. Interestingly, most of these exosomes easily entered the prostatic stromal cells. These results will aid subsequent functional analysis of exosomes.

Most of the existing studies have shown that exosomes participate in the pathophysiological process of various diseases through paracrine and remote secretion as a carrier of signaling molecules. Recently, it was reported in high-profile journals that exosomes are used as carriers to mediate

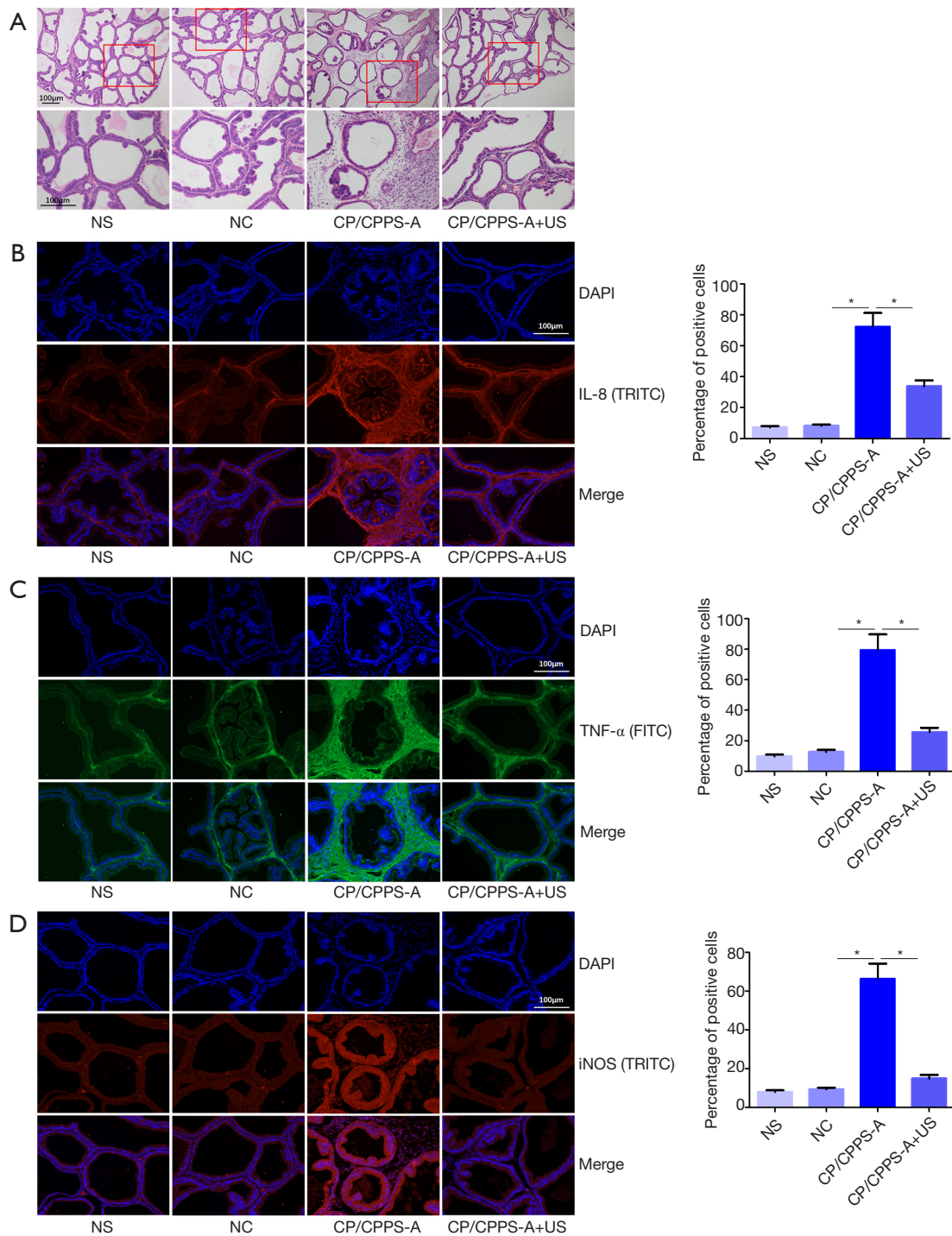


Figure 4 Figure 4 Prostatic fluid exosomes promoted inflammation in prostate tissue. (A) Representative HE-staining images of prostate tissue in SD rats injected with normal saline, NC exosomes, prostatic fluid exosomes of CP/CPSS-A patients, and ultrasound-treated prostatic fluid exosomes of CP/CPSS-A patients. (B) Results of prostate tissue in different groups measured by MPO activity assay. (C–E) Expression of IL-8 (TRITC secondary antibodies), TNF- α (FITC secondary antibodies), and iNOS (TRITC secondary antibodies) in different groups of prostate tissue observed under a fluorescence microscope, scale bar =100 μ m. *, $P < 0.05$. NS, normal saline; NC, normal control; CP/CPSS-A, type IIIA chronic prostatitis/chronic pelvic pain syndrome; CP/CPSS-A + US, ultrasonic treatment in the CP/CPSS-A; MPO, myeloperoxidase.

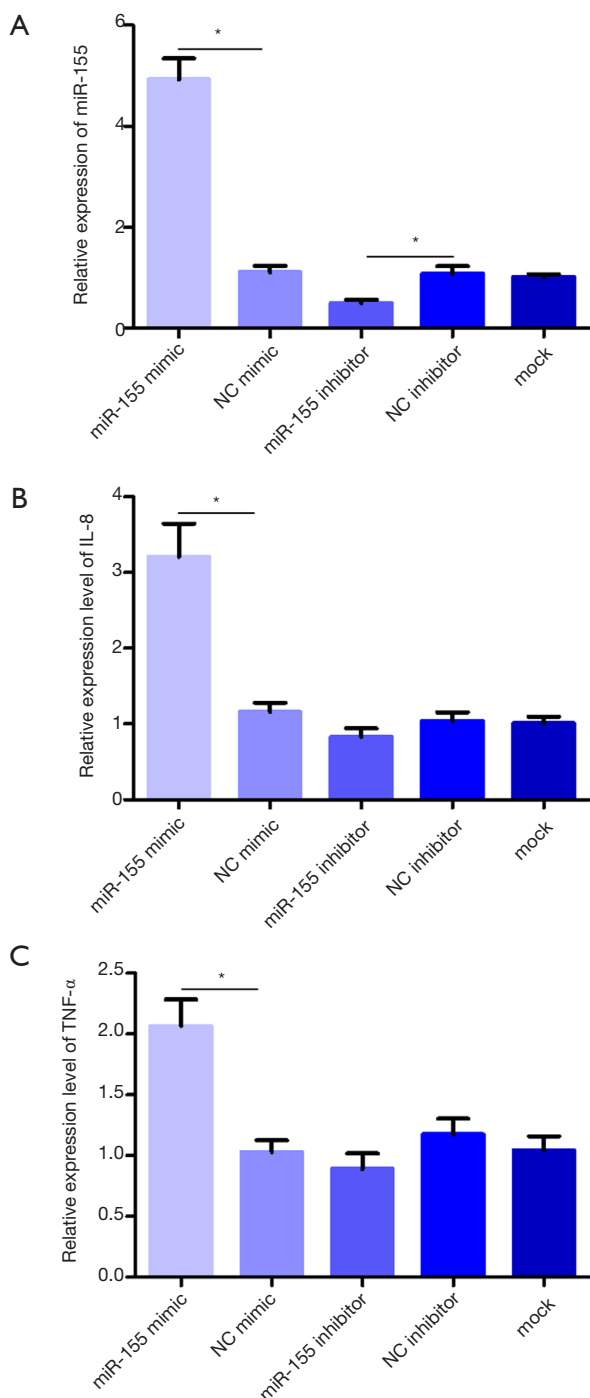


Figure 5 *miRNA-155* promoted inflammation of prostatic stromal cells. (A) Expression of *miRNA-155* in prostatic stromal cells transfected with *miR-155* inhibitor/mimic and negative control inhibitor/mimic measured by qPCR. (B,C) ELISA sequentially measured the relative expression levels of IL-8 and TNF- α in the prostatic stromal cell culture system after regulating the expression of *miRNA-155*. *, $P < 0.05$.

specific miRNA transfer between cells and play a key role in the occurrence and progression of diseases, such as lung injury, diabetes, and vascular dysplasia (22,27,28). Regarding inflammation, exosomes secreted by neutrophils deliver *miRNA-155* to vascular endothelial cells to initiate atherosclerosis and the progression of chronic vascular inflammation (13). For refractory temporomandibular joint osteoarthritis, exosomes secreted by mesenchymal stem cells reduce inflammation and restore matrix homeostasis (15).

miRNA-155 is a highly conserved and important miRNA and has been considered to correlate strongly with inflammatory diseases. Importantly, a recent study has shown that *miRNA-155* may be related to the occurrence of prostatitis, and changes in the level of *miRNA-155* affect the inflammatory response and oxidative stress of autoimmune prostatitis (29), which arouse further interest in the expression of *miRNA-155* in exosomes. In this study, after testing different groups of exosomes, we showed that the concentration of *miRNA-155* in exosomes derived from prostatic fluid in CP/CPPS-A patients was abnormally increased, suggesting that *miRNA-155* was selectively loaded into exosomes and was transported to prostatic stromal cells. These results were consistent with the high-throughput sequencing results of miRNAs in the prostatic fluid of patients with chronic prostatitis reported previously. This study also tested the inflammatory cytokines on the co-culture prostatic stromal cell line and showed that exosomes derived from prostatic fluid in CP/CPPS-A patients promoted the expression of IL-8 and TNF- α . According to previous reports and our results, we believe that exosomes containing high concentrations of *miRNA-155* were a prostatitis-promoting factor.

Exosomes, as macromolecular substances, enter and exit the cell membrane through endocytosis and exocytosis, which require the integrity of the double-layer plasma membrane structure. A previous study has shown that high-frequency ultrasound destroys the plasma membrane structure of exosomes (30), which suggests ways of seeking interventions for pro-inflammatory targets of exosomes. This study used high-frequency ultrasound to process the exosomes derived from the prostatic fluid of CP/CPPS-A patients and used transmission electron microscopy to show that ultrasound treatment destroys most of the exosomal plasma membrane structure. However, the detection of *miRNA-155* did not show any obvious reduction in the miRNA level, indicating that ultrasound only disrupted the exosomal plasma membrane structure but did not affect the exosomes' *miRNA-155* concentration. In further

in vitro experiments, we showed that the pro-inflammatory ability of prostatic fluid exosomes in CP/CPPS-A patients treated with ultrasound was weakened, as manifested by the decreased inflammatory cytokine expression. In *in vivo* experiments, immunofluorescence staining was used to detect the expression of inflammatory cytokines more intuitively in the prostatic fluid exosomes of CP/CPPS-A patients after ultrasound-rupture treatment. The results showed that the expression of inflammatory cytokines in the prostatic fluid exosomes of the CP/CPPS-A + US group was lower than that of the CP/CPPS-A group. The positive expression of IL-8 and TNF- α was mainly localized in the prostate's stroma and mesenchyme, and the positive expression of iNOS was mainly localized in the prostate epithelial cells.

To eliminate the potential interference caused by other substances in exosomes and verify whether *miRNA-155* mimicked exosome-induced prostatitis, we conducted functional analyses of *miRNA-155*. Our results showed that the reduction of *miRNA-155* did not affect IL-8 or TNF- α expression, while simultaneous increases in IL-8 and TNF- α were observed after increasing *miRNA-155*. These results further proved the important role of high *miRNA-155* concentrations in the occurrence and progression of prostatitis.

Conclusions

In this study showed that there were abnormally high concentrations of exosomes in the prostatic fluid of patients with CP/CPPS-A. These exosomes were selectively loaded with *miRNA-155* and were engulfed by prostatic stromal cells in large quantities to activate the immune response and induce inflammation. These results demonstrate a new mechanism in which inflammation may be stimulated in CPPS patients who have WBC in their prostatic fluid. Further study of the signal transduction pathways on which the pro-inflammatory effects of exosomes depend is necessary.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All volunteers included in the study provided written informed consent before participating in this study. The research protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Ethics approval number: KY201801). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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