The purpose of this study was to investigate the effects of ejaculatory duct obstruction (EDO) on contractile efficacy, smooth muscle ultrastructure, and α1A and M3 receptors of rat seminal vesicles (SVs). A total of 48 male rats, aged 14-15 weeks, were randomly divided into three groups, namely, the control, complete EDO, and partial EDO. SV tissues were collected at 4 and 8 weeks postoperatively for further experiments. Results revealed a marked reduction in SV contractile efficacy over time following obstruction in the complete EDO group. The contractile force and frequency decreased and increased in the partial EDO group at week 4, respectively, whereas contractile efficacy significantly reduced at week 8. Moreover, obstruction resulted in significant downregulation in expression of α1A and M3 proteins and mRNAs in rats from the complete EDO group over time. Rats in the partial EDO group initially exhibited an increase followed by a decrease. Analysis of the ultrastructure of SV smooth muscles confirmed the above changes. In conclusion, complete EDO can lead to a progressive decrease in contractile efficiency of SVs. On the other hand, partial EDO can first compensate for the contraction of SVs and gradually decompensate afterwards.

1. Introduction

Infertility affects approximately 15% of the global population of childbearing age, estimated to occur in about 48.5 million couples [1]. Nearly half of these cases are attributed to the male partner [2]. Azoospermia in men is ascribed to two main causes, namely, nonobstructive azoospermia (disorders of spermatogenesis) and obstructive azoospermia (inability to expel normal sperm). Ejaculatory duct obstruction (EDO), a type of obstructive azoospermia that refers to development of abnormal lesions in the ejaculatory duct at the distal end of the seminal tract that obstruct semen expulsion, accounts for 1-5% of all male infertility causes [3]. EDO not only affects fertility, but also causes symptoms, such as weak and painful ejaculation, hemorrhage, perineal pain, and even psychosocial problems [4]. At present, the rapid advancements in imaging and endoscopic technology have improved the diagnosis of EDO. In most cases, the disease can be treated by minimally invasive surgery.

Seminal vesicles (SVs), a pair of sac-like organs composed of an outer smooth muscle layer and the inner subepithelial glandular tissue, are important accessory gonads of the male reproductive system with contractile and secretory functions [5]. SVs, together with the ampulla of the ductus deferens, ejaculatory ducts, and seminal cumulus, form the SV system [6]. Functionally, the SV gland tissue is mainly regulated by parasympathetic nerves, while the smooth muscle layer is regulated by both sympathetic and parasympathetic nerves [7]. In the regulation of the sympathetic pathway, the hypogastric nerve (HN) excites while nerve endings release norepinephrine to excite
α1-adrenoceptor, thereby inducing contraction of SVs [8]. In the parasympathetic regulation pathway, the pelvic nerve (PN) is excited and nerve endings release acetylcholine to activate the muscarinic acetylcholine receptor (mACHR) resulting in the contraction and secretion of SVs [9]. Different receptor subtypes for α1-AR and mACHR have been identified in the SV smooth muscle, of which α1A and M3 are the most dominant [10, 11].

Currently, transurethral resection of the ejaculatory duct (TURED) is the classical surgical modality for treatment of EDO. Results from a study evaluating long-term efficacy of TURED for treatment of EDO-induced male infertility revealed a significant improvement in semen parameters in semen parameters of 63.3% of patients. Consequently, natural pregnancy was achieved in 26.6% of patients, although complete and non-cystic EDO showed poor response [12]. Considering that the surgical treatment did not improve semen parameters and the pregnancy rate of some EDO patients, we hypothesized that EDO might induce secondary changes in SV function.

In the present study, we established an EDO rat model and used it to explore the possible mechanism underlying EDO’s effect on SV contraction. Our findings are expected to provide a theoretical basis for future follow-up treatment for EDO patients who do not respond well to surgical treatment, thereby improving their fertility.

2. Materials and Methods

2.1. Drugs and Reagents. PE and Ach were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China), while analytical reagents, including NaCl, NaHCO3, KCl, CaCl2, KH2PO4, MgSO4, and glucose were bought from Kaitong Chemical Reagent Co., Ltd. (Tianjin, China). Glutaraldehyde and RIPA lysis buffer were obtained from Solarbio (Beijing, China); protease inhibitors were purchased from Sigma-Aldrich, whereas the BCA protein assay kit was acquired from BIOSS (Beijing, China). Primary antibodies targeting α1A, M3, and β-actin were purchased from Beijing Bios Biotechnology Co., Ltd. (α1A, bs-0600R, 1:1000; M3, bs-1289R, 1:500; β-actin, bs-0061R, 1:5000), whereas secondary antibodies polyclonal IRDye 800CW goat anti-rabbit IgG (H+L) (1:20000, 926-32211) were bought from LI-COR. All other reagents were purchased from Servicio technology Co., Ltd. (Wuhan, China) or Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Experimental Animal and Tissue Sources. Forty-eight male Wistar rats, weighing 350–400 g, and aged between 14–15 weeks, were acquired from the Animal Experiment Center of Shanxi Medical University. The animals were randomly divided into three equal groups, namely, complete EDO, partial EDO, and control groups. For rats in the complete EDO group, the SV excratory and ductus deferens were ligated with a 2-0 silk (Jiasheng Medical Supplies Ltd., Suzhou, China). For those in the partial EDO group, a ligation wire was filled with 0.7 mm guide wire, and the ligation degree was unified using a tension transducer (150 g). Rats in the control group were subjected to sham operation, without obstruction of the ejaculatory duct. For the detailed animal modeling protocol and pathology evaluation, please refer to our previous articles [13]. After surgery, rats were housed in an environment maintained at 12-hour light/12-hour dark cycle, a temperature of 23°C with free access to standard diet and clean water ad libitum.

Four weeks after surgery, half the rats in each group were randomly anesthetized with sodium pentobarbital (i.p., 50 mg/kg, Sheng Xing Biotechnology Co., Ltd., Nanjing, China) and subjected to laparotomy. Next, their SVs were carefully dissociated, and a 2-0 silk suture ligated under the knot. For rats in the control group, the silk sutures were ligated in the same position in a similar fashion to those in the experimental group. The SVs were excised from below the new knot and stored at 4°C in Krebs solution (pH 7.4), comprising 118 mM NaCl, 24.8 mM NaHCO3, 4.75 mM KCl, 2.5 mM CaCl2, 1.18 mM KH2PO4, 1.18 mM MgSO4, and 10 mM glucose. The surrounding connective tissue and coagulation glands were also carefully removed. Seminal plasma of SVs of rats in the complete and partial EDO groups was gently squeezed out, and the seminal plasma coagulum was cleaned with forceps. SVs of rats in the control group were punctured using a 1 mL syringe (fitted with a 10 mL syringe needle); the seminal plasma was diluted and aspirated with 4°C Krebs solution without pulling out the needle. After quick processing, the SVs were divided into four parts and stored appropriately. The remaining rats were subjected to the same operation 8 weeks after surgery.

2.3. Organ Bath Studies. Each SV tissue was carefully cut into a 3 mm wide ring, which was subsequently suspended from the triangular-shaped pins and immediately placed in the cavity of a vertical organ bath system containing 10 mL of Krebs solution. The Krebs solution was continuously fed with a gas, comprising 95% O2 and 5% CO2, and kept at 37°C. One pin was fixed to a hook, at the bottom of the bath, while the other was connected to a tension transducer (YP100, Xinhang Electromechanical Equipment Factory, Gaobeidian, China). The rings were equilibrated at 1 g basal tension for 1 hour, with the Krebs solution replaced with fresh one every 20 minutes. Prior to the formal experiment, we selected the SV rings of rats in the control group, and different concentrations of PE and Ach (1 nM–100 μM) were added to the organ bath to determine the optimal contraction-inducing drug concentration. Each time, the rings were incubated for 10 minutes with the drug and then washed twice (5 minutes each time) with Krebs solution. Next, PE and Ach with the optimal concentration for inducing contraction were added to each group of organ baths, respectively, and the contraction tension and frequency were recorded. Variations in isometric contraction of the SV ring were amplified and analyzed using the BL-420F software (Taimeng Software Co., Ltd., Chengdu, China).

2.4. Western Blot Assay. SV tissues were snap-frozen in liquid nitrogen and triturated in RIPA lysis buffer containing protease inhibitors. The lysate was centrifuged at 12,000 rpm for 30 minutes at 4°C, and the supernatant was collected. The concentration of extracted proteins was determined using the BCA kit. The protein samples were mixed with
SDS-PAGE loading buffer and boiled in a water-bath maintained at 100°C for 10 minutes. Equal concentrations of proteins were resolved through the SDS-PAGE and transferred to nitrocellulose membrane using standard techniques. The membranes were blocked with 5% skim milk powder for 1 hour and then incubated overnight with primary antibody diluted in 5% nonfat dry milk at 4°C. Membranes were subsequently washed with TBST and then incubated with the secondary antibody diluted in 5% nonfat dry milk. Target protein bands were detected on the Odyssey Fc Imaging System (LI-COR), and relative protein expression was quantified using ImageJ 1.48v software (National Institutes of Health).

2.5. qRT-PCR. SV tissues were grounded to a fine powder under liquid nitrogen, treated with RNA Extraction kit (Servicebio, Wuhan, China), and homogenized using a KZ-III-F high-speed-microtherm homogenizer (Servicebio, Wuhan, China) to extract RNA. Approximately 2 μg of RNA sample from each experiment was used to synthesize first strand cDNA with the Servicebio® RT First Strand cDNA Synthesis Kit. The cDNA was subjected to qRT-PCR using 2× SYBR Green qPCR Master Mix (Servicebio) targeting the following genes: α1A; forward, 5'-GGCTCTTCTACGTGCCACT-3' and reverse, 5'-AGCGTC ACTTGCTCTGAGTC-3'; M3: forward, 5'-ATCATCACGTT GACCCCCATA-3' and reverse, 5'-ATAGCACACGAGTTCT ACGG-3'; GAPDH: forward, 5'-GCTGAGAATGGGAAGC TGGT-3' and reverse, 5'-GCCCTCTCCATGGTGTTGAA-3'. Amplifications were performed on the ABI StepOnePlus Real-time PCR System under the following conditions: 95°C for 10 minutes (denaturation), 40 cycles of 95°C for 15 seconds (denaturation), and 60°C for 1 minute (annealing and extension). Each sample was quantified at least three times and normalized to internal GAPDH controls. Relative gene expression was calculated using the 2^ΔΔCt method (ΔΔ Ct) = test samples Δ (Ct) — control samples Δ (Ct); Δ (Ct) = test gene (Ct) — GAPDH (Ct).

2.6. Transmission Electron Microscopy (TEM). SV tissues were cut into 1 mm^3 sample blocks and immediately fixed for 2 hours in 4% glutaraldehyde at 4°C. Samples were rinsed three times with 0.1 M phosphate-buffered saline (pH 7.4), 15 minutes each time. They were then fixed for 2 hours in 1% osmic acid in 0.1 M PBS (pH 7.4). Samples were dehydrated in a series of graded ethyl alcohol, treated with acetone, embedded, and finally sectioned to a 70 nm thickness (Leica UC7). Ultrathin sections were stained with uranyl acetate and lead citrate and then visualized on the HT7700 TEM system (Hitachi Ltd., Tokyo, Japan).

2.7. Statistical Analysis. Statistical analyses were performed using SPSS Statistics 26.0 software (IBM, Armonk, New York, USA), and the data were expressed as means ± standard deviations (SD). Differences among multiple groups were compared using one-way analysis of variance (ANOVA), with data followed by p < 0.05 considered as statistically significant.

3. Results

3.1. Effect of PE and Ach on Seminal Vesicle Contraction In Vitro. Some SV rings exhibited spontaneous contractions that almost disappeared after a 1-hour equilibration period. The adrenergic agonist PE and muscarinic agonist Ach potently induced SV ring contractions and caused distinct contraction patterns: tonic and phasic patterns, respectively (Figure 1(a)). The optimal concentration that induced contractions was 100 μM for both PE and Ach. Notably, 100 μM induced maximum contractions of 86% ± 7.62% and 78.5% ± 7.11% for PE and Ach on the calibrated scale, respectively. This concentration was therefore selected for organ bath experiments in the complete and partial groups (Figure 1(b)).

Contraction tension and frequency tended to significantly decrease with increases in obstruction time in both PE- and Ach-induced SV ring contractions in rats in the complete EDO group (p < 0.01), with the exception of PE-induced frequency at 4 weeks in which a slight but statistically insignificant decrease was observed (p = 0.077). Rats in the partial EDO group had slightly (p = 0.077) and significantly (p = 0.013) lower PE- and Ach-induced contractions, respectively, at 4 weeks compared with those in the control group, which were also significantly reduced at 8 weeks (p < 0.01). Rats in the partial EDO group exhibited slightly higher frequency of PE- and Ach-induced contractions compared with those in the control group at 4 (p = 1.00 and p = 0.111), but these changes were significantly reduced at 8 weeks (p < 0.05 and p < 0.01) (Figures 1(c)–1(f)).

3.2. Effect of EDO on α1A and M3 Expressions in Seminal Vesicle Tissues. Rats in the complete EDO group showed lower expression of SV contractile key proteins (α1A and M3) relative to those in the control group, which continued to decrease with the increase in obstruction time. In the partial EDO group, α1A protein was first upregulated at 4 weeks after operation, followed by a significant downregulation at 8 weeks after operation. Similarly, the M3 protein was upregulated 4 weeks after operation, followed by a slight downregulation at 8 weeks, although this was not significant (Figures 2(a) and 2(b)).

3.3. Effect of EDO on α1A and M3 mRNA Expressions in Seminal Vesicle Tissues. The α1A and M3 mRNAs were significantly downregulated in rats in the complete EDO at 4 weeks after surgery, a phenomenon that became more obvious at 8 weeks after surgery, relative to those in the control group. In the partial EDO group, α1A mRNA was slightly and significantly downregulated at 4 and 8 weeks, respectively. On the other hand, M3 mRNA was slightly upregulated at 4 weeks and but downregulated at 8 weeks, although without statistical significance in both cases (Figure 2(c)).

3.4. Seminal Vesicle Smooth Muscle Ultrastructure. The smooth muscle cells of SV in the control group appeared evenly distributed, with a few collagen fibers visible in the small intercellular spaces. Numerous dense plaques, which were continuously distributed, could be seen under the intact sarcolemma. Many myofilaments and normal-shaped organelles, such as
Figure 1: (a) Different traces of seminal vesicle ring contraction induced by PE and Ach. (b) Different concentrations of PE and Ach- (0.01-100 μM) induced seminal vesicle ring contraction in the control group at 4 weeks. (c–f) 100 μM PE- and 100 μM Ach-induced seminal vesicle ring contraction in both complete and partial EDO groups. Results are displayed as a percentage (%) of calibrated scale. The scale range of 0-120% corresponds to 0-1.2 g. *p < 0.05; **p < 0.001 compared to control group at the same time point.
Figure 2: Effects of different degrees and time of EDO on expression levels of α1A and M3 receptors in rat seminal vesicles. (a) Western blots for α1A and M3 proteins, alongside GAPDH used as a control. (b) Protein expression of α1A and M3 was quantified by optical densitometry and normalized to that of GAPDH. (c) qRT-PCR results showing levels of α1A and M3 expressions, alongside GAPDH used as a control. *p < 0.05; **p < 0.001 compared to control group at the same time point.
them were pyknotic and irregularly shaped. Pyknosis and interstitial collagen fibers (CFs) are abundant and partly continuous. Small intercellular space and few collagen fibers (CFs) can be seen around the nucleus (N) (magnification ×2.0 k). (b) Mitochondria (Mit) and a few rough endoplasmic reticulum (rER) with attached ribosomes can be seen in the cytoplasm, a phenomenon that was accompanied by vacuolated mitochondria. At 8 weeks, smooth muscle cells shrank and interstitial collagen fibers proliferated. Moreover, we recorded a reduction in the number and sizes of mitochondria in the cytoplasm, a phenomenon that was accompanied by reduced density of dense plaques. Representative TEM images of SV smooth muscle cells from rats in the complete EDO group are shown in Figure 4. At 4 weeks, there were minor changes compared with the control group, such as increased smooth muscle cell volume and local disturbance of cytoplasmic myofilaments. The number of mitochondria was significantly higher, the structure of the inner mitochondrial ridge was blurred, and some mitochondria were vacuolated. At 8 weeks, smooth muscle cells shrunk and interstitial collagen fibers proliferated. Moreover, we recorded a reduction in the number and sizes of mitochondria in the cytoplasm, a phenomenon that was accompanied by reduced density of dense plaques. Representative TEM images of SV smooth muscle cells from rats in the complete EDO group are presented in Figure 5. At 4 weeks, smooth muscle cell pyknosis and interstitial collagen fiber hyperplasia were observed in the treatment groups. Moreover, mitochondria decreased in the cytoplasm, lobulated, and lysed nuclei. The above changes had been aggravated at 8 weeks. Notably, changes included expansion of the rough endoplasmic reticulum and presence of lipid droplets in the cytoplasm. The number of mitochondria was also significantly reduced, and most of them were pyknotic and irregularly shaped.

4. Discussion

Ejaculation, one of the most essential stages of human fertilization, is divided into two stages: emission and expulsion [14]. Emission refers to secretion of sperm and spermatoplasm from testis and accessory gonads to prostatic urethra [15]. Various enzymes, fructose, and lipids in the spermatoplasm provide energy for sperm. Expulsion refers to the vigorous and rhythmic contraction of sciatic cavernous, bulbar cavernous, and pelvic floor muscles, which results in vigorous expulsion of semen through the urethral opening [16]. EDO, a type of obstructive azoospermia, reportedly seriously affects ejaculation and has been shown to be accompanied by symptoms, such as weak and painful ejaculation, bloody sperm, and perineal pain [4]. The recent rapid advancement in imaging technologies, coupled with the gradual improvement in men’s self-health awareness, have markedly improved EDO diagnosis in patients. In fact, andrology doctors have a common goal to not only effectively treat EDO but also improve male fertility. TURED was first described by Farley and Barnes as a classical surgical modality to improve fertility of EDO patients [17]. The recent rapid advancement of interventional and endoscopic technologies has led to development of transurethral balloon dilation and transurethral seminal vesiculosity (TSV) that have subsequently been applied in clinical practice. Consequently, the latter has been used to treat complications such as hemospermatoz and SV stones during surgery [18, 19].

Over the years, surgery has been used to effectively treat most EDO patients. A previous systematic review of TURED in infertile men with EDO revealed that semen volume improved in 83.0% of patients, while the mean postoperative spontaneous pregnancy rate increased to 25% [20]. Other studies have also demonstrated that the underlying etiology of EDO is an important predictor of surgical success, with partial EDO and cystic EDO reported to confer superior surgical efficacy than complete EDO and inflammatory calcification EDO, respectively [21, 22]. The efficacy of surgical treatment in patients with functional EDO is poor [23]. Some patients do not benefit from surgical removal of the EDO, indicating that the connection of the SVs to the ejaculatory duct is similar to the bladder-urethra structure. Previous studies have shown that bladder outlet obstruction (BOO) can cause dysuria and even decreased renal function. Histologically, this condition manifests as bladder remodeling, such as detrusor cell hypertrophy, atrophy, and accumulation of intercellular collagen fibers [24]. Therefore, we
hypothesized that EDO might lead to SV reconstruction, thereby preventing some EDO patients from benefiting from surgery.

In our previous studies, we successfully established animal models of complete and partial obstruction of the ejaculatory duct [13]. Results indicated that rats in the complete EDO group exhibited progressive SVs, including reduced SV weight, SV atrophy, thinning of smooth muscle layer, and epithelial cell exfoliation. Animals in the partial EDO group exhibited a compensatory mechanism, characterized by increased SV weight and smooth muscle layer thickening, observed at 4 weeks. However, these were decompensated at 8 weeks, as evidenced by gross morphological and histological damage to the SVs. Consequently, we focused on the effects of EDO on contractile function of the isolated SVs, as well as smooth muscle ultrastructure, and changes in α1A and M3 receptors associated with SV contraction.

Results from organ bath experiments, targeting isolated human SV muscle strips, revealed that norepinephrine, phenylephrine, and the vasoconstrictor peptide endothelin 1 were the most effective inducers of contraction, whereas serotonin and adenosine triphosphate were less effective [25]. On the other hand, the muscarinic agonist carbachol only had a little effect [26]. Some previous studies have demonstrated that muscarinic agonist can also induce contraction of SV muscle strips, which was consistent with our experimental results [10, 11]. Lee et al. prepared a ring from isolated SV for the first time and found that it responded better to contraction-inducing drugs and electrical stimulation than the strip preparations [27]. We draw lessons from the SV ring preparation method, which can better retain the SV tubular structure, reduce the operation side damage, and shorten the preparation time.

Results from the SV contraction experiment revealed a reduction in SV contractile efficacy in rats in the complete
EDO group with increase in time of obstruction. In the partial EDO group, we observed a decrease and increase in contraction tension and frequency at 4 weeks, respectively, while the contractile efficacy significantly decreased at 8 weeks. Western blot and qRT-PCR results revealed changes in α1A and M3 receptors that were consistent with results from in vitro SV contraction experiments. It is worth noting that the Ach-induced SV contractile efficiency was significantly lower in the partial EDO group at 8 weeks than in the control, although levels of mRNA and protein expression for α1A and M3 receptors did not significantly decrease. One possible explanation for this phenomenon is that parasympathetic nerves regulate the SV epithelium, while M3 receptors are widely distributed in both the glandular epithelium and smooth muscle layer [7, 10]. TEM examination can explain the above results from an ultrastructural standpoint. SV smooth muscle cells of rats in the complete EDO group appeared damaged, as evidenced by cell pyknosis, collagen fiber hyperplasia, and structural degradation of myofilaments, nucleus, dense plaques, dense bodies, and mitochondria. As the injury worsened, lipid droplets and expanded rough endoplasmic reticulum were observed in the cytoplasm due to prolonged obstruction. These ultrastructural changes can cause smooth muscle cells to lose their normal contractile function, thereby reducing SVs’ ability to contract. In the partial EDO group, we first observed reactivity of SV smooth muscle cells and increased cytoplasmic mitochondrial atrophy, reduced density of dense plaques, and collagen fiber hyperplasia. Prolonging obstruction time resulted in various manifestations, including mitochondrial atrophy, reduced density of dense plaques, and collagen fiber hyperplasia. The above results indicated that complete EDO causes a progressive decline in SV contractile efficiency, while partial EDO first causes SV smooth muscle compensation and reconstruction and followed by decompensation following prolongation of obstruction time. This ultimately affects SV contraction.
This study had several limitations. Firstly, the distance between the ductus deferens and the urethra is relatively small, while the seminal vesicle excretion tube is located in the urethra at the distal end of the ductus deferens. Although local anatomy of SV-vas deference in rats and humans is different, we believe that the EDO rat model is consistent with a blocked semen ejection in human EDO patients, thus does not significantly affect our experimental results. Secondly, SV secretions account for 60 to 70% of the total seminal plasma, which contains a variety of nutrients and biologically active substances that provide nourishment for normal male reproductive activities. SV contraction and secretion are innervated by autonomic nerves. However, we did not evaluate SV secretion function and nerve growth factor in this study. Tsounapi et al. demonstrated that oxidative stress negatively affected SV innervation in a diabetic rat model [28]. In future, we will further evaluate the effect of EDO on secretion function of SVs and autonomic nerve changes, with a view to provide a more comprehensive theoretical basis for treatment of EDO.

5. Conclusion

We have demonstrated the effects of complete and partial EDO on SV contractile efficacy in rats. Notably, analysis of the ultrastructure of smooth muscle as well as expression profiles of α1A and M3 receptors at both transcriptional and translational levels showed that complete EDO caused progressive decline in SV contractile efficiency. Partial EDO not only resulted in a compensatory mechanism of SVs, which was gradually decompensated with extension of EDO time, but also induced injury changes that were similar to those of complete EDO. Taken together, our findings not only provide a rationale to guide future treatment approaches for EDO patients who fail surgical treatment, but also have important implications for improving male fertility worldwide.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The experiments were performed by the Animal Research Ethics Committee of the First Hospital of the Shanxi Medical University. Animals were treated in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors’ Contributions

(I) Conception and design were carried out by J Wang; (II) administrative support was carried out by J Wang; (III) provision of study materials or patients was carried out by J Ren, Z Xing, K Yang, Y Gao, and W Wang; (IV) collection and assembly of data were carried out by J Ren, Z Xing, and Y Ji; (V) data analysis and interpretation were carried out by J Ren, S Fan, Z Wang, and Y Ji; (VI) manuscript writing was carried out by all authors; and (VII) the final approval of the manuscript was carried out by all authors.

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