



# Ozone therapy ameliorates inflammation and endometrial injury in rats with pelvic inflammatory disease



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## ABSTRACT

As a common cause of infertility, pelvic inflammatory disease (PID) is characterized by chronic pain, ectopic pregnancy as well as inflammation and infection of the female upper genital tract. Ozone water, also known as O<sub>3</sub>, has been previously reported to be a distinctly effective agent in treating inflammation. During the present study, we asserted the hypothesis that O<sub>3</sub> could be applied by pelvic inflammation and works to regulate the expression of inflammatory factors including interleukin-6 (IL-6), IL-2 and tumor necrosis factor-α (TNF-α). In an attempt to evaluate the effect of O<sub>3</sub> on PID, an acute PID rat model was subsequently established. O<sub>3</sub> at concentrations of 45 μg/mL and 60 μg/mL in addition to levofloxacin (LVLX) was injected respectively into the PID rats in a bid to alter the contents of inflammatory factors and immunologic markers. Hematoxylin-eosin (HE) staining was applied to analyze endometrial inflammation. Reductions to the contents of IL-6 and TNF-α were recorded, while that of IL-2, IgA, IgG, IgM, C3 and C4, and E rosette formation rate and transformation rate of T lymphocytes exhibited notably elevated levels after the PID rats had been injected with 45 μg/mL O<sub>3</sub>, 60 μg/mL O<sub>3</sub> or LVLX. The pathological condition of the endometrium in rats with PID was alleviated among the PID rats after injected with the 45 μg/mL O<sub>3</sub>, 60 μg/mL O<sub>3</sub> or LVLX. Taken together, the key findings of the current study present evidence demonstrating that the administration of O<sub>3</sub> to the pelvic cavity ameliorated the PID conditions among rat models *via* inhibition of the necrosis of the endometrial epithelial cells as well as alleviated the inflammatory reactions, highlighting a potential novel PID treatment target.

## 1. Introduction

Pelvic inflammatory disease (PID) is commonly occurring condition characterized by infection and inflammation of the female upper genital tract, affecting the fallopian tubes, uterus and adjacent pelvic structures which if not treated appropriately, may spread upward to the peritoneum [1]. The inflammation observed in the condition is predominately driven by the infected host cell which then leads to tissue damage and related reproductive complications observed among PID patients [2]. The later stages of PID, may be accompanied by various complications including tubo-ovarian abscess and pyosalpinx, ectopic pregnancy, infertility, as well as varying degrees of chronic pelvic pain [3]. *Chlamydia trachomatis* and the high prevalence of PID can be attributed to several factors involving sensitive laboratory diagnostics, high-risk sexual behavior as well as the introduction of chlamydia screening programs [4]. The clinical features of PID include lower abdominal

tenderness in both sides, aberrant discharge and bleeding in vagina and cervix, dyspareunia, fever with a body temperature over 38 °C, cervical motion tenderness and adnexal tenderness, with or without palpable lumps [5]. Interstitial cystitis (IC), is a condition generally associated with painful bladder syndrome (PBS), that refers to a chronic inflammation disease occurring in close proximity to the pelvic region [6]. IC/PBS is a disease that is associated with an array of complications including urgent and frequent urinary, pelvic pain, nocturia, pressure and/or discomfort [7,8]. The guideline provided by the Centers for Disease Control and Prevention (CDC) highlights chlamydia trachomatis, neisseria gonorrhoeae, HIV testing and some specific antibiotic regimens as necessary components required for the effective diagnosis and treatment of PID [9]. The combination of levofloxacin (LVLX) and ceftriaxone is a widely applied therapeutic protocol for PID and is even employed in cases of pneumonia caused by drug-resistant isolate, which acts to downregulate inflammation while promoting bacterial clearance

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[10].

Ozone (O<sub>3</sub>) is a colorless gas comprised of 3 oxygen atoms. O<sub>3</sub> is regarded as an energized form of oxygen capable of quickly reacting with other substances [11]. Since its discovery, O<sub>3</sub> has been applied as a therapeutic agent for various diseases, largely due to its ability to prepare the host for the physio-pathological damage brought about by reactive oxygen species [12]. O<sub>3</sub> was initially applied in treatment of post traumatic gangrene of German soldiers during the first world war, during which it was also used to prevent infection in local procedures and to suppress wound infections [13]. O<sub>3</sub> possesses both bactericidal and viricidal properties which can be applied for sterilization, destruction and disinfection of malignant cells [14]. O<sub>3</sub> has anti-inflammatory and antioxidant properties, which are often applied for treating inflammation as a complementary or alternative medicine treatment approach [15,16].

Collectively, the central objective of the current study was to demonstrate the significant inhibition of PID as a result of the administration of O<sub>3</sub> injected through the pelvic cavity. The findings of the study may help clarify the underlying mechanism of O<sub>3</sub> in PID, highlighting its therapeutic value in the treatment of patients with PID.

## 2. Materials and methods

### 2.1. Ethics statement

The study processes and animal operational program were approved by the Animal Ethics Committee of Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University). All efforts were made to reduce the pain of the rats recruited for the experiments in the study, and properly managed after the study.

### 2.2. Study subjects

Before the experiment, 65 Sprague Dawley (SD) female mice (Specific-pathogen-free [SPF] degree; 8~12 weeks; 200~420 g body weight) were provided by Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China; License key: SCXK [Xiang] 2009-0004). Three rats were randomly selected each day for an ultrasound examination before model establishment. The rats were examined consecutively over a period of 7 days.

### 2.3. PID model establishing, grouping and concentration screening of O<sub>3</sub>

Mixed bacteria consisting of staphylococcus aureus (No. 44152), escherichia coli (No. 26002) and M.urealyticum (No. 52011) were used to establish the acute PID female rat model, with the bacterial strains used provided by the Microbiology Department of Hsiang-Ya Medical College (Central South University, Hunan, China). All 65 rats were initially weighed and anaesthetized with 3% pentobarbital sodium (4 mL/kg). Then, 0.3 mL mixed bacterial fluid ( $2 \times 10^{12}$  cells/L; 1 : 1 : 1 dilution) was gently injected into the rats' uterine cavity from cervix uterus. The grinding needle was pulled and drawn in the uterine cavity on several occasions to cause mechanical injury to the endometrial tissues. After that, 0.1 mL bacterial fluid was injected into the uterine cavity near the ovary followed by injecting an additional 0.1 mL bacterial fluid into the uterine cavity on the other side based on the aforementioned procedures. After injection, a gelatin sponge was placed on the cervix to avoid the leakage of the bacterial fluid. On the 15<sup>th</sup> day post modeling, the concentration of O<sub>3</sub> was screened. Five degrees of concentration: 0 µg/mL, 15 µg/mL, 30 µg/mL, 45 µg/mL and 60 µg/mL were set, and 1 mL O<sub>3</sub> at 5 different concentrations was injected respectively into 3 rats. Three days after the injection, the rats went fasting for 12 h, followed by anesthesia with 3% pentobarbital sodium (4 mL/kg) by intraperitoneal injection followed by venous blood collection. IL-6 content in the rat serum was determined, while two appropriate concentrations of O<sub>3</sub> were selected for the following-

experiments.

Fifty PID female rats were selected, and randomly assigned to the control, normal saline (NS), LVLX (PID rats treated by intravenous injection of LVLX), 45 µg/mL O<sub>3</sub> (PID rats injected with 45 µg/mL O<sub>3</sub>) and 60 µg/mL O<sub>3</sub> group (PID rats injected with 60 µg/mL O<sub>3</sub>). O<sub>3</sub> (0.3 mL/d), NS (20 mL/kg/d), and LVLX (0.06 g/kg/d) were respectively injected to the pelvic cavity of PID female rats, while no solution was administered to the rats in the control group. The drug injection was carried out continuously for 7 days, during which one rat was randomly selected from each group for ultrasonic examination and the ultrasonic result obtained after the injection was subsequently compared with the one prior to model establishment. After 7 days, the rats were placed on a fasting program for 12 h, and anaesthetized with 3% pentobarbital sodium (4 mL/kg) via intraperitoneal injection before they were killed and their venous blood was obtained. The uterine tissues of the rats in each group were collected and certain uterus tissues selected and fixed in 10% neutral formalin solution. After 24 h, the tissue samples were dehydrated in gradient ethanol and embedded with paraffin. The remaining tissue samples were preserved with liquid nitrogen for later use.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The contents of IL-6, TNF-α and intercellular cell adhesion molecule-1 (ICAM-1) in rat serum were detected using ELISA based on the following procedures. Firstly, the venous blood of the rats was obtained, stood still for 1 h, and centrifuged at 1610 × g for 10 min with the supernatant collected and preserved at -20 °C. ELISA kit (kit number of TNF-α, IL-6 and IL-2 was 69-25328, 69-40133 and 69-40092; brought from Wuhan Merschk Biological Technology Co., Ltd., [Hubei, China]) was employed to detect the sample serum in accordance with the kit instructions. The detection process was conducted three times.

### 2.5. Detection of immunological indexes

At the 24<sup>th</sup> h after the final injection, the rats were anaesthetized and their blood was collected from the abdominal aorta. The content of immunoglobulin G (IgG), IgA and IgM, and complement C<sub>3</sub> and C<sub>4</sub> was then measured by single-radial-immunodiffusion (SRD), with the transformation rate and E rosette formation rate of T lymphocyte examined using the acid-α-naphthyl-acetate esterase (ANAE) staining method.

### 2.6. Hematoxylin-eosin (HE) staining

Seven days post model establishment, the paraffin-embedded rat uterus tissues were collected from each group and later sliced into 5 µm serial sections. After extension and collection at 45 °C, the sections were baked at 60 °C for 1 h before they were dewaxed by xylene. The sections were then hydrated, routinely stained with HE (Beijing Solarbio Science and Technology Co., Ltd, Beijing, China), dehydrated in an ascending order of ethanol series, and cleared in xylene. Later, the damage and repair condition of the uterus tissues were observed under an optical microscope (XP-330, Shanghai Bing Yu Optical Instrument Co. Ltd., Shanghai, China) after they had been sealed with neutral balsam. The uterus condition was scored on a 0~4 point system, depending on the lesion extent of the endometrial epithelial cell: uterus with no lesion was equal to 0 points; uterus with very mild lesion was given 1 point; uterus with mild lesion extended under 1/3 was granted 2 points; uterus with moderate lesion extended between 1/3 ~ 2/3 was given 3 points; and uterus with severe lesion extended over 2/3 was marked as 4 points. According to the standard of inflammatory cell infiltration [17], uterus with no inflammatory cellular infiltration was marked as 0 points; uterus with slight inflammatory cell infiltration was given 1 point; uterus with slight infiltration shown in mucoderm was marked as 2 points; uterus with infiltration shown in muscle layer was granted 3

points; and uterus with infiltration shown in all layers was marked as 4 points. After tallying the points, the score of rats in each group was calculated. The higher the score was, the greater the severity of the PID lesion.

## 2.7. Immunohistochemistry

The uterus tissues of rats in each group were obtained 7 days after model establishment, after which they were fixed in 10% formalin, and dehydrated in an ascending order of ethanol series and embedded in paraffin after 24 h. Twelve days later, the tissue blocks were cut into 5 slices, each section being 3–4  $\mu\text{m}$ . After routine dewaxing and hydration, the sample sections were dewaxed respectively with xylene I and xylene II for 10 min, dehydrated with 100% graded ethanol for 2 min, 95% for 2 min, 80% for 2 min, and 70% for 2 min. The sample sections were then washed with phosphate buffer saline (PBS) (twice, 5 min each), soaked in 3%  $\text{H}_2\text{O}_2$  for 10 min, washed with PBS (twice, 5 min each), followed by high pressure antigen repair for 90 s. After cooling to room temperature, the sections were washed with PBS, and added with 5% bovine serum albumin (BSA) for incubation at 37 °C for 30 min. Based on the aforementioned procedure, 50  $\mu\text{L}$  rabbit anti-rat monoclonal antibody (IL-6, 1 : 100, ab7737, Abcam Inc., Cambridge, MA, USA) was added to the sections and incubated at 4 °C overnight. After 2 min of PBS washing, 50  $\mu\text{L}$  of biotinylated rat anti-goat IgG (SF8-0.3, 1 : 100, Beijing Solarbio Science & Technology Co., Ltd. [Beijing, China]) was added to the sections and incubated at 37 °C for 30 min, which were added with spontaneous alternation behavior (SAB) and colored with diaminobenzidine (DAB). Later, the sections were restained with hematoxylin for 5 min, washed with tap water for 10 min, dehydrated, cleared, sealed, and placed under a microscope for section observation purposes, with PBS employed as the primary antibody. Normal brownish-yellow cells were reflective of the IL-6 protein positive cells, and the positive expression rate of IL-6 = the number of positive cells/total cells  $\times$  100%. The experiments were repeated three times.

## 2.8. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

The uterus tissues were collected, added with 1 mL Trizol kit (Invitrogen, Carlsbad, CA, USA), and ground during ice bathing. Total RNA was extracted from the rat apical tissues based on the Trizol instructions. The concentration and purity of the total RNA were detected by means of ultraviolet spectrophotometry (UV1901, Shanghai Austria Science Instrument Co., Ltd., Shanghai, China), ensuring that  $\text{A}_{260}/\text{A}_{280} = 1.8\text{--}2.0$ . The sample concentration was adjusted to 50 ng/ $\mu\text{L}$ , and the RNA was reversely transcribed to cDNA (50 ng/ $\mu\text{L}$ ) using PrimeScript™ RT reagent kit (Takara, RR047A, Beijing Think-Far Technology Co., Ltd., Beijing, China). Then, 10  $\mu\text{L}$  reverse transcription system was performed according to the kit instructions and the reaction conditions were set as follows: reverse transcription at 37 °C for 45 min, reverse transcriptase inactivation at 85 °C for 5 min, cryopreservation at -80 °C for further use. Primers were design by a Premier 5.0 software and synthesized by Beijing TsingKe Biological Technology Co. Ltd., (Beijing, China) (Table 1). The synthesis was performed in ABI 7900 HT RT-qPCR instrument (ABI 7900, Shanghai PuDi Biotech Co., Ltd., [Shanghai, China]) based on two-step method.  $\beta$ -actin was regarded as the internal reference, while the reaction conditions were as follows: pre-denaturation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s and extension at 72 °C for 15 s. The relative mRNA expression levels of IL-6, ICAM-1, and TNF- $\alpha$  were calculated based on the  $2^{-\Delta\Delta\text{Ct}}$  method. Three duplicate wells were set in each gene of each sample, and the experiments were repeated three times.

**Table 1**  
Primer sequences of related genes by RT-qPCR.

Gene	Sequences
IL-6	Forward: 5'-GAGAAAAGAGTTGTGCAATGGC -3' Reverse: 5'- ACTAGGTTTGCCGAGTAGACC -3'
IL-2	Forward: 5'- CAGCATGCAGCTCCGATC -3' Reverse: 5'- GTGGGTGCGCTGTGACA -3'
TNF- $\alpha$	Forward: 5'- TGCCTCAGCCTCTTCTCATT -3' Reverse: 5'- CCCATTTGGGAACCTTCTCCT -3'
$\beta$ -actin	Forward: 5'- ACCACCATGTACCCAGGCATT -3' Reverse: 5'- CCACACAGAGTACTTGGCGCTC -3'

Notes: RT-qPCR, reverse-transcription quantitative polymerase chain reaction; IL-6, interleukin 6; IL-2, interleukin 2; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

## 2.9. Western blot analysis

Initially, 30 mg of tissue samples were collected from each group respectively, ground into homogeneous powder at low temperature and washed with PBS three times. After adding with protein lysate and placing on ice for 20 min, the lysate was centrifuged at 25,764g for 20 min. The supernatant was collected after centrifugation and the purity of the total protein was determined using a bicinchoninic acid (BCA) protein detection kit (P0012-1, Beyotime Biotechnology Co., Shanghai, China). Next, 50  $\mu\text{g}$  of protein was collected, dissolved in 2  $\times$  sodium dodecyl sulfate (SDS) loading buffer and boiled at 100 °C for 5 min. Then, the samples in each group subsequently underwent electrophoresis in 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto poly vinylidene fluoride (PVDF) membranes and sealed with 5% dried skimmed milk at room temperature for 1 h. After being washed with PBS for 2 min, the PVDF membranes were incubated with the primary rabbit anti-rat IL-6 (1 : 20, ab7737), IL-2 (1 : 2000, ab180780), TNF- $\alpha$  (1 : 2000, ab6671) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1 : 2000, ab8245) followed by washing with Tris-buffered saline Tween-20 (TBST) solution. The experiment was repeated three times in a consecutive manner. Later, horseradish peroxidase (HRP) labeled goat anti-rabbit secondary antibody was diluted into density of 1 : 5000 and incubated with the membrane for 1 h. After that, the membranes were washed with TBST three consecutive times (5 min each time). Finally, the membranes were colored using enhanced electroluminescence (ECL), exposed by X ray and photographed. The optical density (OD) value of each colored band was analyzed by Gel imaging system. The relative protein content of sample = average OD/average OD of the corresponding internal reference. The experiments were repeated three times, and the protein content of each sample was recorded and analyzed.

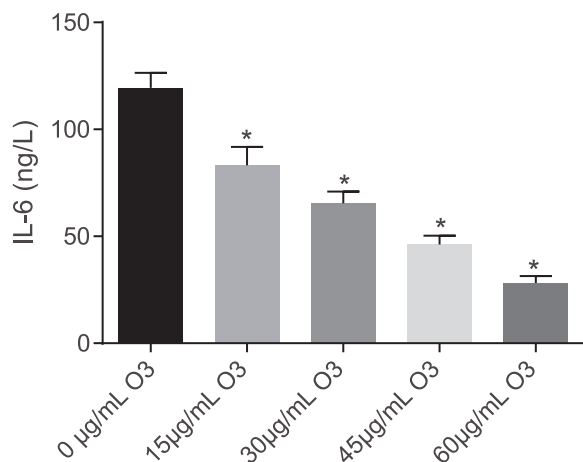
## 2.10. Statistical analysis

All experimental data were analyzed by SPSS 18.0 (IBM Corp, Armonk, NY, USA). Measurement data were expressed as mean  $\pm$  standard deviation. Comparisons between two groups were analyzed by *t*-test, while the comparisons among multi-groups were analyzed by one-way analysis of variance.  $p < 0.05$  was indicative of statistical significance.

## 3. Results

### 3.1. IL-6 serum level in PID rats decreases with the increase of $\text{O}_3$ concentration

The serum of PID rats was treated with  $\text{O}_3$  at varying concentrations in order to observe their varied influence in the IL-6 content. The selected concentration results of  $\text{O}_3$  are depicted in Fig. 1. The results indicated the IL-6 content was decreased in the PID rats after treatment  $\text{O}_3$  with different concentrations. The higher the concentration was, the



**Fig. 1.** The serum level of IL-6 in the PID rats is reduced after treatment with 0 µg/mL, 15 µg/mL, 30 µg/mL, 45 µg/mL and 60 µg/mL O<sub>3</sub> \*,  $p < 0.05$  vs. 0 µg/mL O<sub>3</sub> group,  $n = 10$ ; experimental result value was the measurement data, expressed as mean ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments were independently repeated 3 times; IL-6, interleukin 6; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone.

lower the IL-6 content detected. The PID rat serum treated with 0 µg/mL O<sub>3</sub> when compared to the serum treated with other concentrations had significantly reduced IL-6 content ( $p < 0.05$ ,  $n = 10$ ); the serum treated with 45 µg/mL and 60 µg/mL O<sub>3</sub> had the lowest IL-6 content. Thus, 45 µg/mL O<sub>3</sub> and 60 µg/mL O<sub>3</sub> were selected for the subsequent experiments. These results revealed that O<sub>3</sub> injected into the PID rats diminished the IL-6 content, among which the 45 µg/mL and 60 µg/mL O<sub>3</sub> had the most obvious effects.

**3.2. Improved ultrasonic examination results of PID rats after injection with 45 µg/mL or 60 µg/mL O<sub>3</sub>**

Ultrasonography is often employed to aid in the diagnosis of PID and direct PID treatment (Fig. 2.). Before modeling, no apparent abnormal lesions were detected in the pelvic cavity of rats. After

**Table 2**  
The serum levels of TNF-α, IL-6 reduce and that of IL-2 elevates in PID rats after injected with 45 µg/mL or 60 µg/mL O<sub>3</sub>.

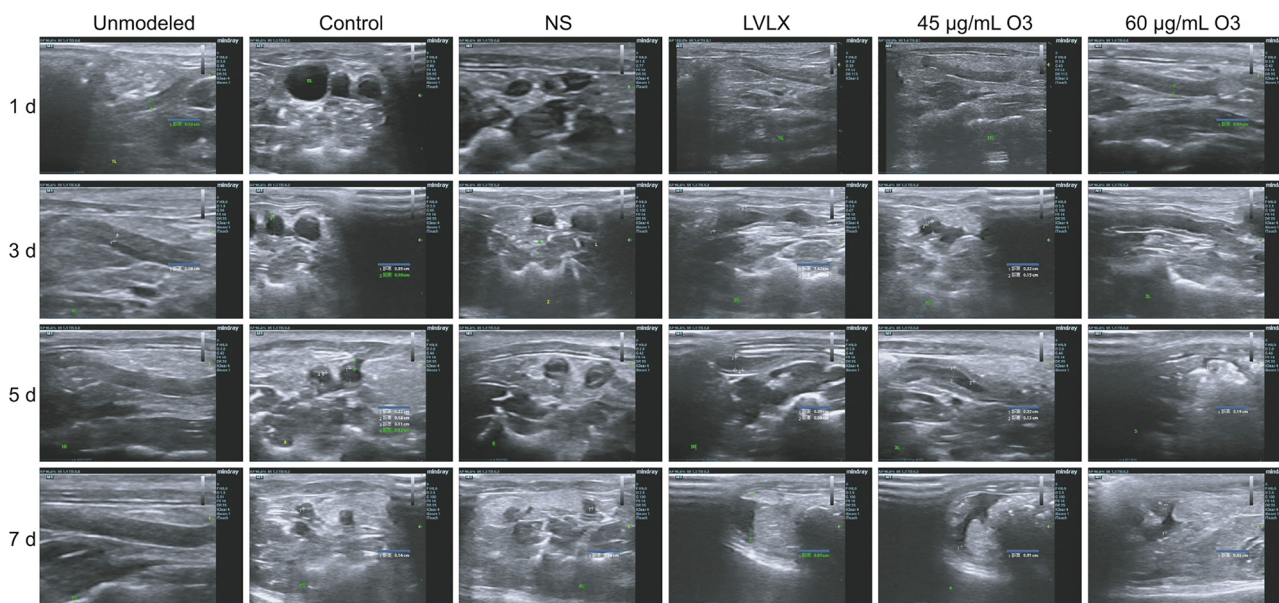
Groups	TNF-α (ng/L)	IL-6 (ng/L)	IL-2 (ng/L)
Control	108.67 ± 3.45	83.20 ± 3.19	79.21 ± 2.66
NS	105.25 ± 4.12	86.78 ± 3.94	75.53 ± 4.69
LVLX	52.02 ± 1.99*	46.12 ± 4.11*	109.62 ± 1.86*
45 ug/mL O <sub>3</sub>	49.34 ± 3.79*	48.52 ± 2.21*	108.50 ± 3.49*
60 ug/mL O <sub>3</sub>	50.23 ± 2.19*	44.12 ± 4.59*	111.51 ± 2.58*

Notes: TNF-α, tumor necrosis factor-alpha; IL-6, interleukin 6; IL-2, interleukin 2; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone; NS, normal saline; LVLX, Levofloxacin; \*,  $p < 0.05$  vs. the control group,  $n = 10$ ; experimental result value was the measurement data, which were shown as mean ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments carried out 3 times independence detection.

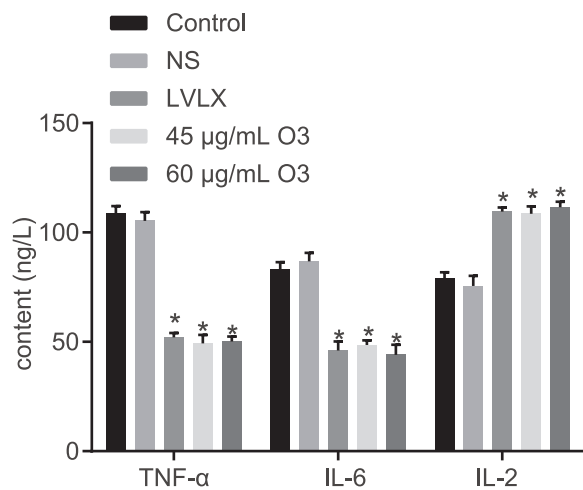
successful modeling, full mold focus display showed that the lump boundary and capsule of PID rats’ pelvic cavity were obscure. Dense light spots were observed inside the capsule with weak or strong echo. Imaging of cystic lesions revealed the pelvic cavity in elliptical with a clear boundary. Inside the sack, a fluid dark area could be observed with light spots, echo, and rough cyst wall. Imaging of mixed lesions showed a round or oval shape with a relatively clear boundary and disorder echo. The echo showed cystic or solid changes inside the sack. After treatment with 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> or LVLX, the ultrasound imaging significantly improved while the imaging of the pelvic cavity in the NS and control group exhibited no notable difference. These results indicated that O<sub>3</sub> leads to pathological improvement of PID in rats.

**3.3. The content of TNF-α and IL-6 decreases while that of IL-2 increases in PID rats injected with 45 µg/mL or 60 µg/mL O<sub>3</sub>**

The effect of O<sub>3</sub> in terms of regulating the contents of TNF-α, IL-6 and IL-2 in PID rats was analyzed using ELISA (Table 2 and Fig. 3). Compared with the control group, no significant difference was detected in regard to the contents of inflammatory factor TNF-α, IL-6 and IL-2 in the NS group ( $p > 0.05$ ,  $n = 10$ ). The contents of TNF-α and IL-6 decreased, while that of IL-2 increased in the 45 µg/mL O<sub>3</sub> group or



**Fig. 2.** O<sub>3</sub> conduces to the pathological improvement of PID rats observed by ultrasonography. In pelvic cavity of successfully modeled rats, full mold focus display showed that the lump boundary and capsule were obscure. The ultrasound imaging of PID rats was significantly improved after treatment with 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> or LVLX. PID, pelvic inflammatory disease; NS, normal saline; LVLX, Levofloxacin; O<sub>3</sub>, ozone.



**Fig. 3.** The serum levels of IL-6 and TNF-α in the PID rats serum are reduced after treated by 45 µg/mL or 60 µg/mL O<sub>3</sub>, while that of IL-2 is increased. TNF-α, tumor necrosis factor-alpha; IL-6, interleukin 6; IL-2, interleukin 2; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone; NS, normal saline; LVLX, Levofloxacin; \*, *p* < 0.05 vs. the control group; *n* = 10; experimental result value was the measurement data, expressed as ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments were independently repeated 3 times.

60 µg/mL O<sub>3</sub> group (*p* > 0.05, *n* = 10). These results provided evidence suggesting that O<sub>3</sub> inhibits the development of inflammation of PID rats.

**3.4. Immunoglobulin content and immunologic function of PID rats are ameliorated after injected with 45 µg/mL or 60 µg/mL O<sub>3</sub>**

The effect of O<sub>3</sub> on the content of IgG, IgA and IgM, C<sub>3</sub> and C<sub>4</sub>, and the E rosette formation rate and transformation rate of T lymphocyte in each group were determined (Tables 3 and 4). No significant difference was detected between the control and NS group in relation to the immunoglobulin and immune function of rats (*p* > 0.05, *n* = 10). The contents of IgG, IgA and IgM, C<sub>3</sub> and C<sub>4</sub>, and the E rosette formation rate and transformation rate of T lymphocyte in rats in the 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> and LVLX groups were markedly increased (*p* < 0.05, *n* = 10). These results indicated that O<sub>3</sub> improves the immunoglobulin content and immune function of PID rats.

**3.5. Endometrial injury in PID rats is reduced after injected with 45 µg/mL or 60 µg/mL O<sub>3</sub>**

The effect of O<sub>3</sub> on the endometrial injury of PID rats was assessed

**Table 3**

The levels of IgG, IgA and IgM increased in rats with PID after injected with 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> or LVLX.

Groups	IgG (g/L)	IgA (g/L)	IgM (g/L)
Control	0.43 ± 0.06	0.28 ± 0.03	0.55 ± 0.04
NS	0.45 ± 0.02	0.33 ± 0.04	0.54 ± 0.02
LVLX	0.66 ± 0.06*	0.47 ± 0.08*	0.73 ± 0.04*
45 µg/mL O <sub>3</sub>	0.62 ± 0.05*	0.52 ± 0.05*	0.83 ± 0.07*
60 µg/mL O <sub>3</sub>	0.67 ± 0.05*	0.55 ± 0.04*	0.87 ± 0.06*

*Notes:* IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone; NS, normal saline; LVLX, Levofloxacin; \*, *p* < 0.05 vs. the control group, *n* = 10; experimental result value was the measurement data, which were shown as mean ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments carried out 3 times independence detection.

**Table 4**

E rosette formation rate and transformation rate of T lymphocyte are decreased after PID rats injected with 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> or LVLX.

Groups	T lymphocytes		Alexin (ng/L)	
	Transformation rate (%)	E rosette formation rate (%)	C3	C4
Control	45.65 ± 1.36	16.61 ± 2.41	0.31 ± 0.02	0.03 ± 0.01
NS	46.51 ± 2.14	18.23 ± 2.01	0.28 ± 0.07	0.04 ± 0.01
LVLX	67.35 ± 2.69*	30.52 ± 3.16*	1.29 ± 0.32*	0.21 ± 0.03*
45 µg/mL O <sub>3</sub>	75.53 ± 1.12*	26.67 ± 2.07*	0.82 ± 0.17*	0.29 ± 0.04*
60 µg/mL O <sub>3</sub>	79.56 ± 4.38*	34.88 ± 3.79*	1.03 ± 0.15*	0.23 ± 0.02*

*Notes:* PID, pelvic inflammatory disease; NS, normal saline; LVLX, Levofloxacin; O<sub>3</sub>, ozone; \*, *p* < 0.05 vs. 0 µg/mL O<sub>3</sub> group; *n* = 10; experimental result value was the measurement data, which were shown as mean ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments carried out 3 times independence detection.

**Table 5**

O<sub>3</sub> inhibits the inflammatory injury of endometrium of PID rats.

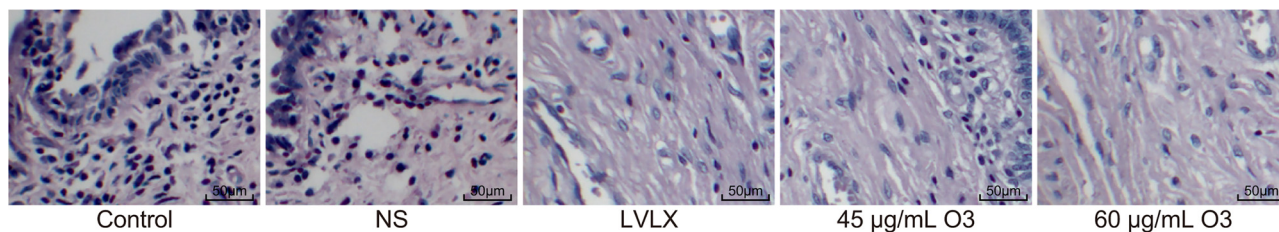
Groups	n	Comprehensive score of pathology
Control	10	2.70 ± 0.70
NS	10	3.20 ± 0.60
LVLX	10	1.80 ± 0.90*
45 µg/mL O <sub>3</sub>	10	1.20 ± 0.60*
60 µg/mL O <sub>3</sub>	10	1.50 ± 0.70*

*Notes:* PID, pelvic inflammatory disease; NS, normal saline; LVLX, Levofloxacin; O<sub>3</sub>, ozone; \*, *p* < 0.05 vs. the control group; *n* = 10; experimental result value was the measurement data, which were shown as mean ± standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments carried out 3 times independence detection.

through the application of HE staining (Table 5 and Fig. 4). Compared with the control group, the inflammation of endometrium of rats in the 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> and LVLX groups was significantly improved. Scattered or layered inflammatory cell infiltration in addition to necrosis and degeneration of the epithelial cells was observed among the rats in the NS and control groups. Vague epithelial hyperplasia and thickening of the endometria were observed in rats administered with 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> and LVLX groups, with a clear dividing line between the mucous layer and the myometrium with a largely integrated epithelial structure, signs of exfoliation and necrosis in the myometrium, and a large amount of inflammatory infiltration in each layer. These results suggested that O<sub>3</sub> inhibits the inflammatory injury of the endometrium in rats with PID.

**3.6. IL-6 positive expression in the uterus tissue of PID rats is reduced after injected with 45 µg/mL or 60 µg/mL O<sub>3</sub>**

The inhibitory effect of O<sub>3</sub> on IL-6 positive expression in the uterus tissue of PID rat was detected by immunohistochemistry methods. Compared with the control group, no significant difference was observed in relation to the IL-6 positive expression in the uterus tissue of rats in the NS group (*p* > 0.05, *n* = 10). The IL-6 positive expression in the uterus tissue of rats in the 45 µg/mL O<sub>3</sub>, 60 µg/mL O<sub>3</sub> and LVLX groups was significantly decreased (*p* < 0.05, *n* = 10). The results obtained indicated that O<sub>3</sub> inhibits IL-6 positive expression in the uterus tissue of PID rats (Fig. 5A-B).



**Fig. 4.** O<sub>3</sub> inhibits the inflammatory injury of endometrium of PID rats observed by HE staining (the original magnification is  $\times 200$ ). Endometrium PID rats shows scattered or layered inflammatory cell infiltration and the necrosis and degeneration of epithelial cells while the symptoms are relieved after rats are injected with 45  $\mu\text{g/mL}$  O<sub>3</sub>, 60  $\mu\text{g/mL}$  O<sub>3</sub> or LVLX. HE, Hematoxylin-eosin; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone.

**3.7. The expression of IL-6 and TNF- $\alpha$  in the uterus tissue of PID rats is inhibited while the expression of IL-2 is increased following the injection of 45  $\mu\text{g/mL}$  or 60  $\mu\text{g/mL}$  O<sub>3</sub>**

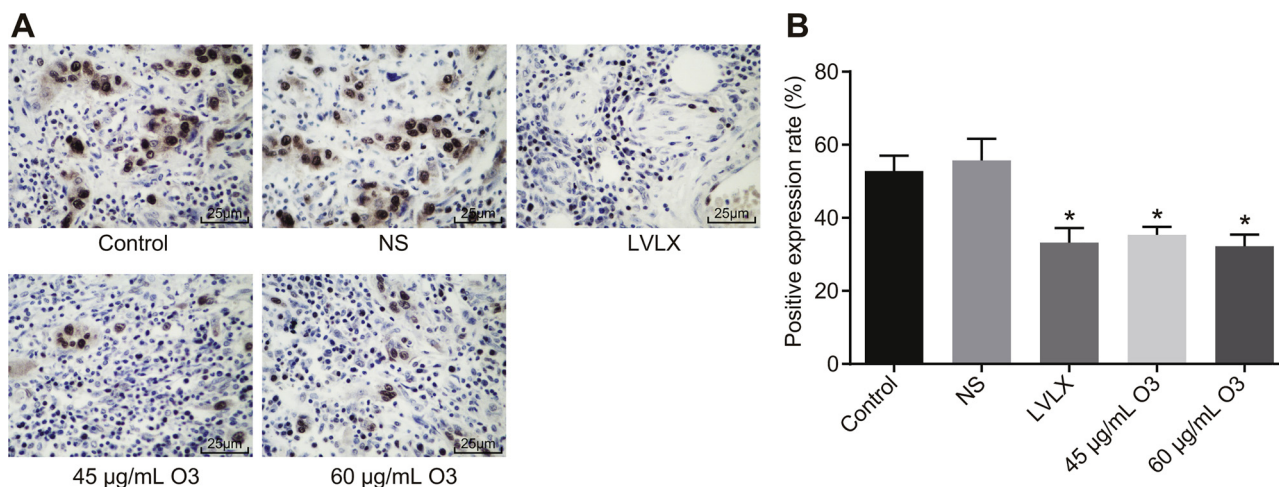
The inhibitory effect of O<sub>3</sub> on the expression of IL-6, IL-2 and TNF- $\alpha$  in the uterus tissue of PID rats was detected using RT-qPCR and western blot analysis methods (Fig. 6). No significant difference was detected between rats in the control and NS group regarding the mRNA and protein expression of IL-6, IL-2 and TNF- $\alpha$  in the uterus tissues ( $p > 0.05$ ,  $n = 10$ ). The mRNA and protein expression of IL-6 and TNF- $\alpha$  in the uterus tissue of rats in the 45  $\mu\text{g/mL}$  O<sub>3</sub>, 60  $\mu\text{g/mL}$  O<sub>3</sub> and LVLX groups significantly decreased, while that of IL-2 significantly increased (all  $p < 0.05$ ,  $n = 10$ ). These results indicated that O<sub>3</sub> inhibits the inflammation-related expression the uterus tissue of PID rats.

#### 4. Discussion

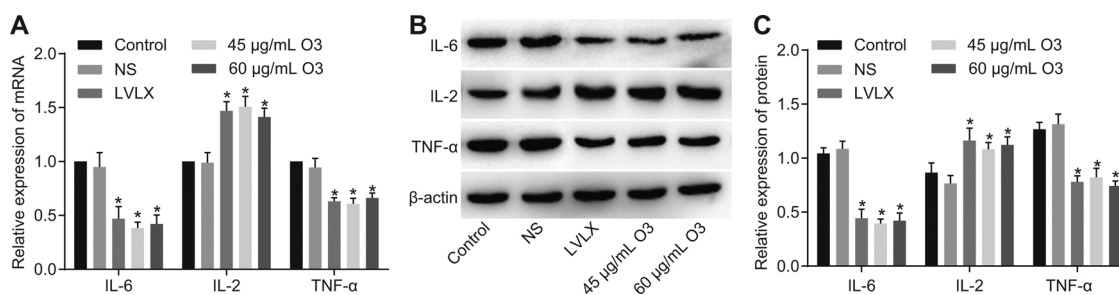
As an infection commonly affecting the female lower reproductive tract, PID if not adequately treated may spread to the upper reproductive tract [18], leading to severe reproductive complications including chronic pelvic pain, infertility and ectopic pregnancy [19]. Currently, treatment guidelines place significant emphasis on eradicating the infection by means of a regimen of broad-spectrum antibiotics while surgical action is restricted to drainage of pelvic collections and early division of adhesions [2]. During the present study, O<sub>3</sub> was identified as a potential facilitator in alleviating the development of PID by means of pelvic cavity injection.

A key result obtained in the current study revealed that the contents of IL-6 and TNF- $\alpha$  in PID rats were decreased while that of IL-2

increased after the administration of O<sub>3</sub>, especially after the administration of 45  $\mu\text{g/mL}$  and 60  $\mu\text{g/mL}$  O<sub>3</sub>. O<sub>3</sub> therapy has been reported to prevent cyclophosphamide-induced hemorrhagic cystitis through diminishing inflammation, bladder oxidative stress and nitric oxide level [20]. Reducing joint injury and inflammation, decreasing of pro-inflammatory cytokines, TNF- $\alpha$  as well as IL-1 $\beta$  transcripts and the re-establishment of cellular redox balances after O<sub>3</sub> therapy have been demonstrated in cases of rheumatoid arthritis [21]. O<sub>3</sub> has been suggested to be effective as hyperbaric oxygen in reducing inflammatory cytokine and oxidative parameter [22]. Optimized O<sub>3</sub> therapy after a brief exposure of blood to O<sub>3</sub> has been speculated to be the potential solution capable of reprogramming the immune system that may ultimately keep HIV at bay [23]. IL-6 is a typical cytokine which features pleiotropic activity and redundancy [24]. IL-6 blockade can inhibit acute T helper type 17 response and ameliorate the chronic inflammation through its differentiation [25]. TNF- $\alpha$  is a pro-inflammatory cytokine known to influence the pathogenic mechanisms of various immune-mediated or inflammatory diseases [26]. TNF- $\alpha$  release is regulated by TNF- $\alpha$  converting enzyme which cuts membrane-bound TNF- $\alpha$  to shed its ectodomain as a soluble cytokine [27]. Moreover, endometriosis is a disease leading to chronic pelvic pain, dysmenorrhea, infertility/sterility [28], and in endometriosis, the correlation among TNF- $\alpha$ , cytokines, Fas ligand (FasL/CD95 L), and transforming growth factor  $\beta$  (TGF- $\beta$ ) could make the balance translated into the signaling pathway promoting apoptosis [29]. Upregulated sFasL in endometriosis has been previously hypothesized to contribute to FasL activity decline and mononuclear cells containing mFas may become novel targets serving as killer of endometriotic cells expressing FasL [30]. During the initial presentation of endometriosis, elevated



**Fig. 5.** IL-6 positive expression is reduced in the uterus tissue of PID rats after injected with 45  $\mu\text{g/mL}$  O<sub>3</sub>, 60  $\mu\text{g/mL}$  O<sub>3</sub> or LVLX. Panel A and B, immunohistochemical staining of the uterus tissue of rats after injected with 45  $\mu\text{g/mL}$  O<sub>3</sub>, 60  $\mu\text{g/mL}$  O<sub>3</sub> and LVLX (the original magnification is  $\times 400$ ); \*,  $p < 0.05$  vs. the control group;  $n = 10$ ; experimental result value was the measurement data, expressed as mean  $\pm$  standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments were independently repeated 3 times; IL-6, interleukin 6; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone water; NS, normal saline; LVLX, Levofloxacin.



**Fig. 6.** The expression of IL-6 and TNF- $\alpha$  in the uterus tissue of PID rats is downregulated while that of IL-2 is upregulated by injection of 45  $\mu\text{g/mL}$  and 60  $\mu\text{g/mL}$  O<sub>3</sub>. Panel A, RT-qPCR revealed that the relative mRNA expression of IL-6 and TNF- $\alpha$  in the uterus tissue of PID rats decreased while that of IL-2 increased after injection with 45  $\mu\text{g/mL}$  and 60  $\mu\text{g/mL}$  O<sub>3</sub>; Panel B and C, Western blot analysis demonstrates that the GAPDH proteins of IL-6 and TNF- $\alpha$  in the uterus tissue of PID rats were inhibited by injection of 45  $\mu\text{g/mL}$  and 60  $\mu\text{g/mL}$  O<sub>3</sub> while those of IL-2 increased; \*,  $p < 0.05$  vs. the control group;  $n = 10$ ; experimental result value was the measurement data, expressed as mean  $\pm$  standard deviation, and the comparisons among multi-groups were analyzed by one-way analysis of variance; all experiments were independently repeated 3 times; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin 6; IL-2, interleukin 2; PID, pelvic inflammatory disease; O<sub>3</sub>, ozone.

TNFR1 and sTNF- $\alpha$  has been reported to possibly induce the responses of inflammation while in moderate and severe stages, high levels of TNFR2 and mTNF- $\alpha$  would induce death process [31]. Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), which is a toxicant exerting specifically inhibitory effect on TGF- $\beta$  in endometriosis is very likely to cause the inflammatory-like endometrial microenvironment [32]. IL-2 is a critical element in the maintenance and development of T regulatory cells and the death of the activation-induced cells, which aids to inhibit inappropriate immune reaction in addition to mediating immune tolerance [33]. Interestingly, a recent study evaluated the influence of O<sub>3</sub> on inflammation in multiple sclerosis patients and found that O<sub>3</sub> was able to decrease TNF- $\alpha$ , IL-1 $\beta$ , and increase IL-10 serum levels, which was consistent with the results of the current study [34].

Another finding of this study indicated that O<sub>3</sub> can effectively improve the blood immunoglobulin content and immune function in rats with PID. IgG mediates pro- and anti-inflammatory activities through the engagement of its Fc fragment with distinct Fc $\gamma$  receptors [35]. A previous study suggested that IgG molecules modulate T and B cell responses, suggesting that IgG is a primary factor in the establishment and maintenance of the immune homeostasis [36]. IgA regulates microbiota composition and mediates intestinal homeostasis [37]. Mustafaev EM et al. demonstrated that the concentrations of IgG, IgM, IgA were all high after O<sub>3</sub> therapy [38]. As a multifunctional protein, component C<sub>3</sub> is widely acknowledged to interact explicitly with over 10 different cell surface receptors or plasma proteins [39]. Complement C<sub>3</sub> was previously identified as an autoantigen involved in the development of inflammatory disease [40]. Complement component C<sub>4</sub> is a central protein in the classical and lectin pathways [41]. A previous study concluded that C<sub>4</sub>, as a member of the classical activation pathway of the complement system, plays an important part in autoimmunity [42]. T lymphocytes (Tregs) control immune responses by suppressing various inflammatory cells [43]. During the current study, the contents of IgG, IgA and IgM, C<sub>3</sub> and C<sub>4</sub>, and the E rosette formation rate and transformation rate of T lymphocyte all exhibited marked increases after the PID rat models had been injected with 45  $\mu\text{g/mL}$  O<sub>3</sub>, 60  $\mu\text{g/mL}$  O<sub>3</sub> or LVLX. These results indicated that O<sub>3</sub> improves the content of immunoglobulin and immune function of rats with PID.

Taken together, the key findings of the current study provide evidence suggesting that O<sub>3</sub> may aid in the treatment of PID and can function as a promising therapeutic agent for inflammatory diseases. Additional studies are required in order to further demonstrate the effects of O<sub>3</sub> on the inhibition of PID as well as to further investigate the side effects of O<sub>3</sub> in order for it to be considered as an official therapy for PID. We look forward to determining whether there is a time-dependent effect of O<sub>3</sub> on PID in the future studies.

## Conflict of interest

None.

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