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The NLRP3 inflammasome is a potential target of ozone therapy aiming to ease chronic renal inflammation in chronic kidney disease

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ABSTRACT

Ozone therapy is an effective medical treatment for various diseases. A previous study has demonstrated its reno-protective effect in chronic kidney disease (CKD), but the mechanism involved is not completely known. This study produced the 5/6 nephrectomized CKD rat model and investigated whether the reno-protective effect of ozone therapy was achieved by its anti-inflammatory property through the modulation of the NLRP3 inflammasome. The results showed that ozone therapy at a low concentration improved renal function and ameliorated renal morphological injury in 5/6 nephrectomized rats. The expression of NLRP3, ASC, and caspase-1-p10 in the kidney of these rats was simultaneously lowered by ozone therapy. Moreover, renal inflammation caused by IL-1β was significantly alleviated by ozone therapy. The Pearson correlation analysis indicated that the protein level of IL-1β was positively correlated with renal injury scores. Taken together, these results indicated that ozone therapy might reduce sterile renal inflammation and slow down CKD progression through the modulation of the NLRP3 inflammasome in 5/6 nephrectomized rats.

1. Introduction

Progressive chronic kidney disease (CKD), an irreversible disease ending with end-stage renal failure, is induced and gradually aggravated by constant glomerular and tubulointerstitial injuries with a wide spectrum of causes such as diabetes and hypertension [1]. It has caused worldwide concern in public health for many years, and the health burden due to it is still rising [2]. Thus far, despite expensive renal replacement therapy, few cost-effective therapeutic measures have been developed to ameliorate this chronic renal injury and slow down the progression of CKD [3].

Sterile renal inflammation is considered as the central therapeutic target to reduce chronic renal injury and slow down the progression of CKD [4]. This persistent inflammation leads to progressive renal fibrosis and the gradual loss of renal function, which accelerates CKD progression [5]. Nacht domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) inflammasome is the most well-studied inflammasome that is recognized to play a crucial part in the initiation and continuance of inflammation in various diseases [6]. It is well known that external or internal warning signals can activate the NLRP3 inflammasome via pattern recognition receptor NLRP3. This results in the oligomerization of NLRP3 with the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which leads to the proteolytic cleavage of caspase-1. Once cleaved, caspase-1 modulates the maturation of IL-1β and leads to inflammation [7]. The NLRP3 inflammasome alerts the immune system to combat insults, but contributes to pathological injury if constantly activated. The NLRP3 inflammasome has recently aroused great interest among many nephrologists because of its contribution to sterile renal inflammation in CKD [8–10]. Targeting the NLRP3 inflammasome is therefore a promising strategy to reduce renal inflammation in CKD.

Ozone, a strong oxidant, was once used as a potent gaseous sterilant. Interestingly, modern medicine has proved that proper application of ozone-oxygen mixture at low concentration, also known as ozone therapy, could effectively improve organ ischemia-reperfusion, herniated disks and skin ulcers in the clinic [11–13]. After being studied for many years, ozone therapy has been applied by many institutes as a beneficial and cost-effective alternative medical measure, and it has become increasingly popular around the world [14,15]. Emerging evidence indicates that improving the antioxidant system and inducing oxidative-stress adaptation are the main mechanisms for successful ozone therapy in various diseases [16,17]. Recent advances revealed that ozone therapy could exert an anti-inflammatory action through the regulation of NF-κB in liver ischemia-reperfusion injury and rheumatoid arthritis. In addition, it can ameliorate renal inflammation through the modulation of TLR4 in acute renal ischemia-reperfusion injury [11,18,19]. However, it is still unknown whether ozone therapy

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could exert an anti-inflammatory effect to reduce chronic renal injury in CKD by regulating the NLRP3 inflammasome. Thus, we developed the 5/6 nephrectomized rat model and aimed to address the issue.

2. Materials and methods

2.1. Animals and experimental design

Male Sprague-Dawley rats weighing about 180–200 g (8 weeks of age) were obtained from the Animal Experimental Center of Xiangya School of Medicine, Changsha, China. The rats were housed in a room with a stable temperature of 22 °C–23 °C, humidity at 50%–60% and a 12-hour light and dark cycle for 7 days of acclimation. The rats were fed a standard diet, and water was freely available. All the experimental procedures were approved by the Experimental Animal Center Committee in Xiangya School of Medicine and the procedure was performed in accordance with international animal care guidelines.

The 2-step 5/6 nephrectomy was conducted as reported previously [20]. Briefly, the rats were anesthetized with pentobarbital (40 mg/kg). Then, a surgical operation through the retroperitoneal approach was carried out to excise 2 poles of the left kidney and hemostatic absorbable collagen sponges were applied to stop bleeding. After 1-week recovery, the right kidney was removed.

After another 1-week recovery from the second surgery, experimental rats received ozone therapy once a day, which continued for 2 weeks. Ozone therapy was conducted by referring to previous research [21], using a medical ozonator (YKS-1000g, Ikou Co., Ltd., Zhuhai, China) generating 3% ozone/oxygen gas mixture. The ozone concentration measured by a spectrophotometer in the ozonator was set at 50 μg/mL. After determining the body weight of the rats, the therapeutic dose was set at 1.1 mg/kg, which has been proved to be safe and effective in previous experiments [22]. In addition, the gas mixture was administered by rectal insufflation with the application of a polyethylene cannula.

The rats were divided into 3 groups: (1) Control group ($n = 10$) rats received a sham operation, (2) CKD group ($n = 10$) rats received 5/6 nephrectomy but no treatment, and (3) OT group ($n = 10$) rats received 5/6 nephrectomy, and received ozone therapy. The experiment was terminated at week 10, a suitable time point to study chronic renal injury [23]. All the rats were sacrificed 24 h after ozone therapy. Blood and kidney samples were collected for further analysis.

2.2. Biochemical analysis

Serum creatinine (Cr), urea nitrogen (BUN) and electrolytes including potassium (K), inorganic phosphorus (IP) and ionized calcium (Ca) were measured by standard techniques using an Olympus AU 2700 Analyzer (Olympus Optical Co., Ltd., Tokyo, Japan).

2.3. Pathological analysis

The kidney was immersed immediately after dissection in 4% paraformaldehyde for fixation and embedded in paraffin 24 h later. Slides of kidney sections at 3 μm thickness were stained with hematoxylin–eosin (H&E), Masson trichrome (MT), and Periodic Acid-Schiff (PAS), and then assessed under a microscope. Referring to previous research [24], glomerulosclerosis was evaluated in 50 glomeruli on each PAS-stained section using a semiquantitative score as follows: 0, no sclerosis; 1, sclerosis changes in <25% of the glomeruli; 2, sclerosis changes in 25% to 50%; 3, sclerosis changes in 51% to 75%; and 4, sclerosis changes in >75%. Tubulointerstitial injury was assessed in 15 fields on each MT-stained section using Image-Pro Plus 6.0 software. The extent of tubulointerstitial injury was assessed by counting the percentage of areas with tubular dilation and interstitial fibrosis per field of cortex. Scores from 0 to 4 were used as follows: 0, normal interstitium; 1, <25% of areas injured; 2, 26% to 50% of areas injured; 3, 51% to 75% of areas injured; 4, >75% of areas injured. All the evaluations were performed by an experienced pathologist masked to the specimens.

Immunohistochemical staining was performed as follows. In brief, the paraffin section slides were dewaxed in xylene and rehydrated. Hydrogen peroxide (3%) was applied to abolish endogenous hydrogen peroxide. The slides were sequentially retrieved using an antigen microwave retrieval technique and blocked with 10% normal goat serum. The slides were incubated with primary antibodies against NLRP3 (Santa Cruz) at 4 °C overnight. After washing 3 times with PBS, the slides were incubated with HRP-conjugated antibody at room temperature for 30 min. Diaminobenzidine was used to color the slides. Staining was controlled under the microscope, and the reaction was terminated by washing with distilled water. The slides were counterstained with hematoxylin and assessed under a microscope. A semi-quantitative analysis was performed by detecting optical density in 15 randomly selected cortical fields for each section using Image-Pro plus 6.0 software [25].

2.4. Western blot analysis

Total and nuclear proteins were extracted from the frozen kidney by using EpiQuik™ protein extraction kit (Epigentek) according to the manufacturer's instructions and quantified by the bicinchoninic acid method. The extracted proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, and the membranes were blocked with 5% non-fat milk in TBST buffer. The membranes were incubated with primary antibodies against NLRP3 (Abcam), ASC (Abcam), caspase-1-p10 (Santa Cruz), p-NF-κB P65 (Santa Cruz), pro-IL-1β and cleaved IL-1β (Abcam) overnight at 4 °C. After rinsing 3 times with TBST, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase (ZSGB-BIO, Beijing, China) for 1 h at room temperature. The membranes were then rinsed 4 times. An enhanced chemiluminescence detection kit was applied to visualize specific bands. Quantity One software was employed to detect optical densities. The results are presented as ratios of the target protein to H3 protein and actin protein and assessed. In addition, β-actin was used as a control for determining the purity of nuclear protein.

2.5. Real-time PCR analysis

Total RNA was extracted from frozen kidney using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was detected by a spectrophotometer. Reverse transcription was performed with a cDNA synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. A SYBR Green mix kit (Applied Biosystems, CA, USA) was applied in the PCR. A total of 25 μL of the PCR reaction mixture contained 12.5 μL of SYBR Green mix, 2 μL of cDNA (2×), 1 μL of the forward primer, 1 μL of the reverse primer, and 8.5 μL of ddH_2O. The primers used are listed in Table 1, and GAPDH was used as a reference gene. PCR was performed with a Gene Cycler (Bio-Rad, CA, USA) under the following cycling conditions: initial denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s,

Table 1
Primer list.

Gene	Sequence	Length
NLRP3	Forward: 5′-TCTTTGCGGCTATGTACTATCT-3′ Reverse: 5′-TTCTAATAGGACCTTCACGT-3′	113 bp
IL-1β	Forward: 5′-CTGTGACTCGTGGGATGATG-3′ Reverse: 5′-AGGGATTTTGTCGTTGCTTG-3′	211 bp
IL-6	Forward: 5′-AGTTGCCTTCTTGGGACTGA-3′ Reverse: 5′-ACAGTGCATCATCGCTGTTC-3′	218 bp
TNF-α	Forward: 5′-TGCCTCAGCCTCTTCTCATT-3′ Reverse: 5′-GGGCTTGTCACTCGAGTTTT-3′	180 bp

extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. Data were presented as the mRNA ratio of the target gene to GAPDH.

2.6. Statistics analysis

SPSS 17.0 software was used for statistics analysis. The data are presented as mean ± SD. One-way analysis of variance Student–Newman–Keuls test was implemented to compare the means of the different groups. Pearson correlation analysis was applied to evaluate the correlation between the protein level of cleaved-IL-1β and renal injury scores, including the glomerulosclerosis score and tubulointerstitial injury score in rats. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Ozone therapy improved renal function

Serum Cr, BUN, K, Ca, and IP were measured to evaluate the renal function of the experimental rats. Compared with the Sham group, the CKD group showed significantly higher Cr, BUN, K, and IP levels (Table 2, $P < 0.05$) but a slightly lower Ca level (Table 2, $P > 0.05$). However, the OT group showed significantly lower Cr, BUN, K, and IP levels after ozone therapy, relative to those of the CKD group (Table 2, $P < 0.05$). But instead of a significant difference, there was only an increasing trend towards a higher Ca level in the OT group relative to that of the CKD group (Table 2, $P > 0.05$). The inconsistency of serum Ca level in CKD rats might be associated with some compensatory mechanisms in rats [26] or the additional effect of ozone therapy on other Ca-regulating hormones, for example, parathyroid hormone. These results suggested that ozone therapy improved the renal function of 5/6 nephrectomized rats.

3.2. Ozone therapy improved renal morphologic injury

Staining with H&E, MT and PAS was performed to detect renal morphologic changes in experimental rats. Staining with H&E showed that the CKD group had developed serious renal injury, as evidenced by enlargement of the glomerulus, thick glomerular basement membrane, tubular expansion, loss of brush border of proximal tubules, tubular atrophy and serious inflammatory-cell infiltration, while renal injury was moderate in the OT group (Fig. 1A). Consistently, MT staining and PAS staining also indicated severe glomerular sclerosis and tubulointerstitial fibrosis in the CKD group while injury was moderate in the OT group (Fig. 1A). The semi-quantitative analysis of MT staining and PAS staining showed that the injury scores of glomerular and tubulointerstitium in the CKD group were significantly higher than those in the control group (Fig. 1B, $P < 0.05$) while the OT group showed significantly lower scores than those in the CKD group (Fig. 1B, $P < 0.05$). Taken together, these results indicated that ozone therapy could improve chronic glomerular and tubulointerstitium injury in 5/6 nephrectomized rats.

Table 2
Effects of ozone therapy on renal function of experiment rats.

Serum biochemical indexes	Control group	CKD group	OT group
Cr (um/L)	29.22 ± 4.79	125.70 ± 22.16#	86.90 ± 18.89*
BUN (um/L)	5.68 ± 2.02	26.89 ± 7.47#	16.64 ± 6.24*
K (mmol/L)	4.61 ± 0.32	6.03 ± 0.41#	5.45 ± 0.45*
Ca (mg/dL)	2.39 ± 0.13	2.20 ± 0.27##	2.38 ± 0.21**
IP (mg/dL)	2.44 ± 0.18	2.82 ± 0.46#	2.42 ± 0.30*

Date was represented by mean ± SD ($n = 10$). Cr: creatinine; BUN: blood urea nitrogen; IP: inorganic phosphorus.
# $P < 0.05$, vs control group.
$P > 0.05$, vs control group.
* $P < 0.05$, vs CKD group.
** $P > 0.05$, vs CKD group.

3.3. Ozone therapy regulated the NLRP3 inflammasome in the kidney of 5/6 nephrectomized rats

To investigate whether the reno-protective action of ozone therapy was associated with the modulation of the NLRP3 inflammasome, NLRP3, ASC and caspase-1-p10 were measured in the kidney of experimental rats. Immunohistochemistry results indicated that NLRP3 was expressed not only in glomeruli but also in the tubulointerstitium, mainly located in the proximal tubules, of 5/6 nephrectomized rats (Fig. 2A). The semi-quantitative analysis indicated that the CKD group showed significantly higher NLRP3 expression both in the glomeruli and tubulointerstitium compared with the Control group (Fig. 2A, $P < 0.05$) while the expression in the OT group was lower than that in the CKD group (Fig. 2A, $P < 0.05$). These results indicated that ozone therapy might down-regulate the NLRP3 inflammasome in the kidney of 5/6 nephrectomized rats.

To further confirm this conclusion, western blotting was performed to determine the protein levels of NLRP3, ASC and cleaved caspase-1 in the kidney of experimental rats. As compared with the Sham group, the CKD group showed a higher protein and mRNA expression of NLRP3 (Fig. 2B, $P < 0.05$). Treated with ozone therapy, the OT group showed a lower NLRP3 level than that in the CKD group (Fig. 2B, $P < 0.05$). Consistently, the protein levels of ASC and caspase-1-p10 were higher in the CKD group than those in the Sham group, whereas ozone therapy significantly lowered those of the OT group (Fig. 2C, $P < 0.05$).

3.4. Ozone therapy lowered IL-1β, IL-6 and TNF-α levels

This study investigated the infiltration of IL-1β, a central inflammatory cytokine as well as a crucial downstream target of the NLRP3 inflammasome, in the kidney of experimental rats. The inflammation-inducing activity of IL-1β is generally achieved by cleaved IL-1β, which is cleaved from its inactive precursor pro-IL-1β [27]. The results showed that rats in the CKD group had a higher protein expression of pro-IL-1β and cleaved IL-1β relative to those of the Sham group (Fig. 3A, $P < 0.05$). By contrast, rats in the OT group (with ozone therapy) had lower protein levels of pro-IL-1β and cleaved IL-1β than those in the CKD group (Fig. 3A, $P < 0.05$). The RT-PCR results also showed that the mRNA levels of IL-1β, IL-6 and TNF-α were significantly lowered by ozone therapy in the OT group compared with the CKD group (Fig. 3B, $P < 0.05$). These results indicated anti-inflammatory activity of ozone therapy in 5/6 nephrectomized rats.

3.5. Ozone therapy suppressed P-NF-κB P65 activation

This study detected nuclear p-NF-κB P65, a key upstream regulator which up-regulates pro-IL-1β [27], in the kidney of experimental rats. The results showed that the protein level of p-NF-κB P65 was higher in the CKD group, relative to that of the Sham group, whereas ozone therapy significantly lowered the p-NF-κB P65 level in the OT group (Fig. 3C, $P < 0.05$).

3.6. Positive correlations existed between cleaved-IL-1β and renal injury scores

Pearson correlation analysis showed that the protein level of IL-1β was positively correlated with renal injury scores including the glomerulosclerosis score ($r = 0.6714$, $P < 0.05$) and tubulointerstitial injury score ($r = 0.5667$, $P < 0.05$) in the kidney of rats in the CKD group and OT group (Fig. 4).

4. Discussion

The 5/6 nephrectomized rat model is considered as a classic animal model for CKD research. After the surgical operation was performed, the remnant kidney of the experimental rat would suffer high perfusion,

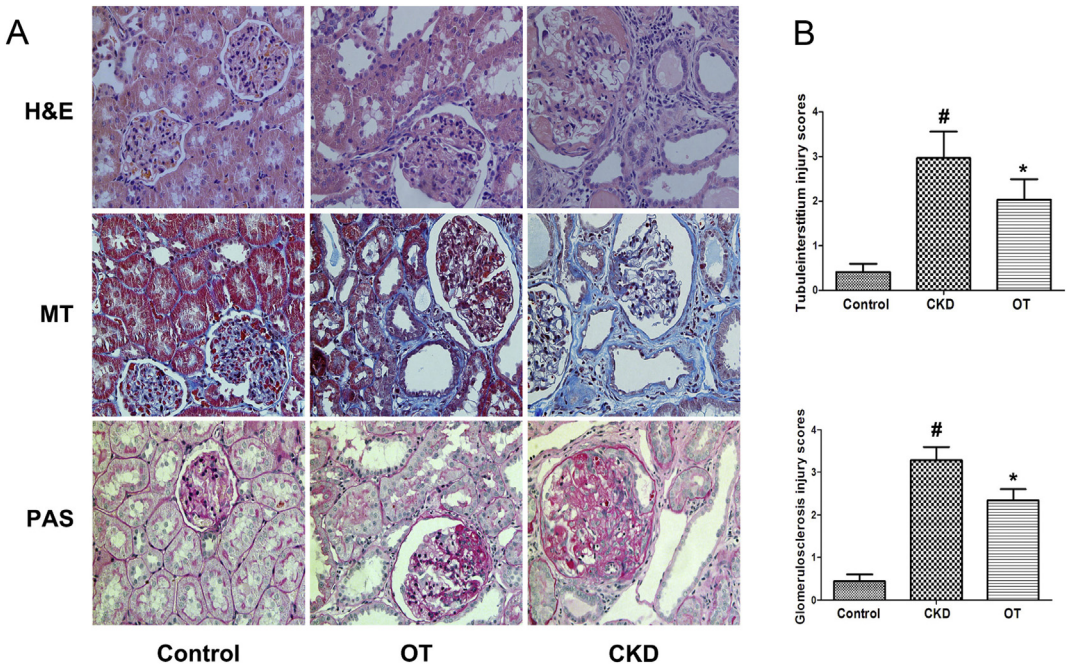

Fig. 1. Effect of ozone therapy on renal morphology at week 10. (A) Representative images showing H&E, MT and PAS staining of different groups (original magnification, ×400). (B) Semi-quantitative analysis of MT staining (Tubulointerstitium injury scores) and PAS staining (Glomerulosclerosis injury scores) of different groups. Each value is expressed as mean ± SD (n = 10). $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs CKD group. H&E, hematoxylin and eosin staining; MT, Masson trichrome staining; PAS, Periodic Acid-Schiff staining.

high pressure, as well as high filtration, gradually leading to the functional deterioration of residual nephrons. This would result in chronic glomerular and tubulointerstitial injuries. These changes are similar to those found in CKD patients in the clinic [23,28]. In the present study, we produced 5/6 nephrectomized rats, which were euthanized and studied at week 10 of the experiment: this was a suitable time point to investigate renal injury [23]. From the study results, 5/6 nephrectomized rats suffered from serious deterioration of renal

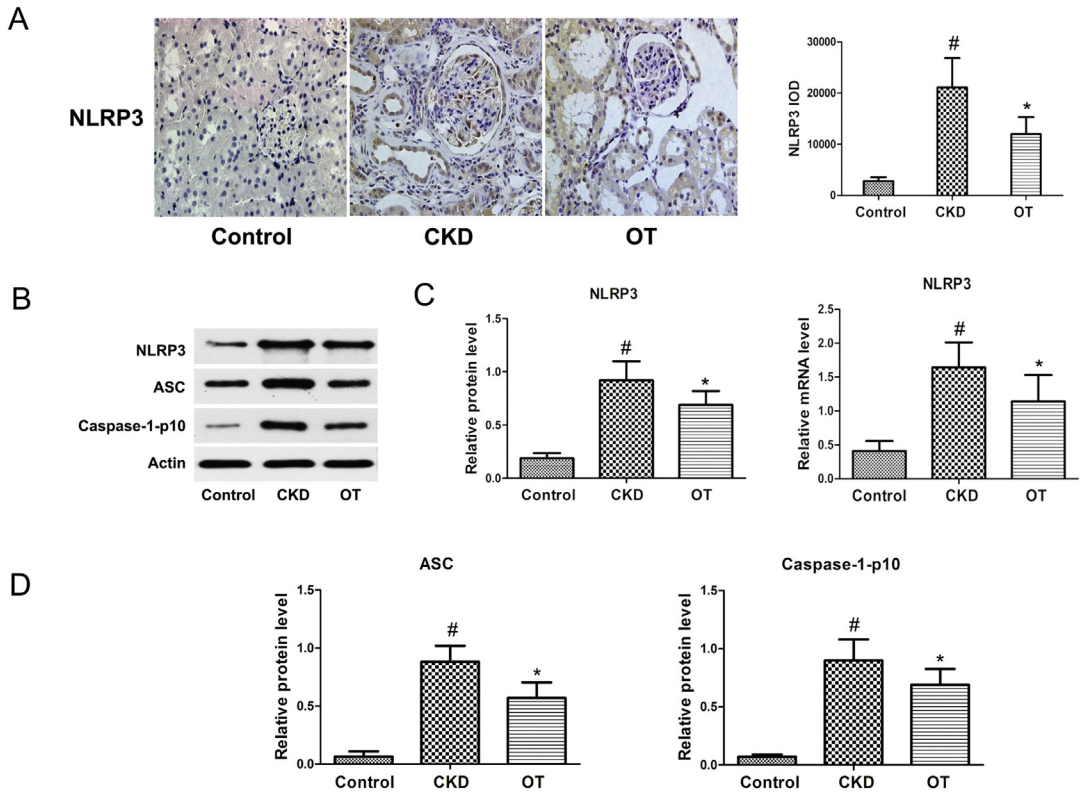

Fig. 2. Effects of ozone therapy on the NLRP3 inflammasome in the kidney of experimental rats. (A) Representative immunohistochemistry images (original magnification, ×400) and semi-quantitative analysis of NLRP3 staining. (B) Representative Western blot images of NLRP3, ASC, caspase-1-p10 and actin. (C) Bar graph showing the quantification of the immunoreactive bands of NLRP3 and NLRP3 mRNA levels. (D) Bar graph showing the quantification of the immunoreactive bands of ASC and caspase-1-p10. Each value is expressed as mean ± SD (n = 10). $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs CKD group.

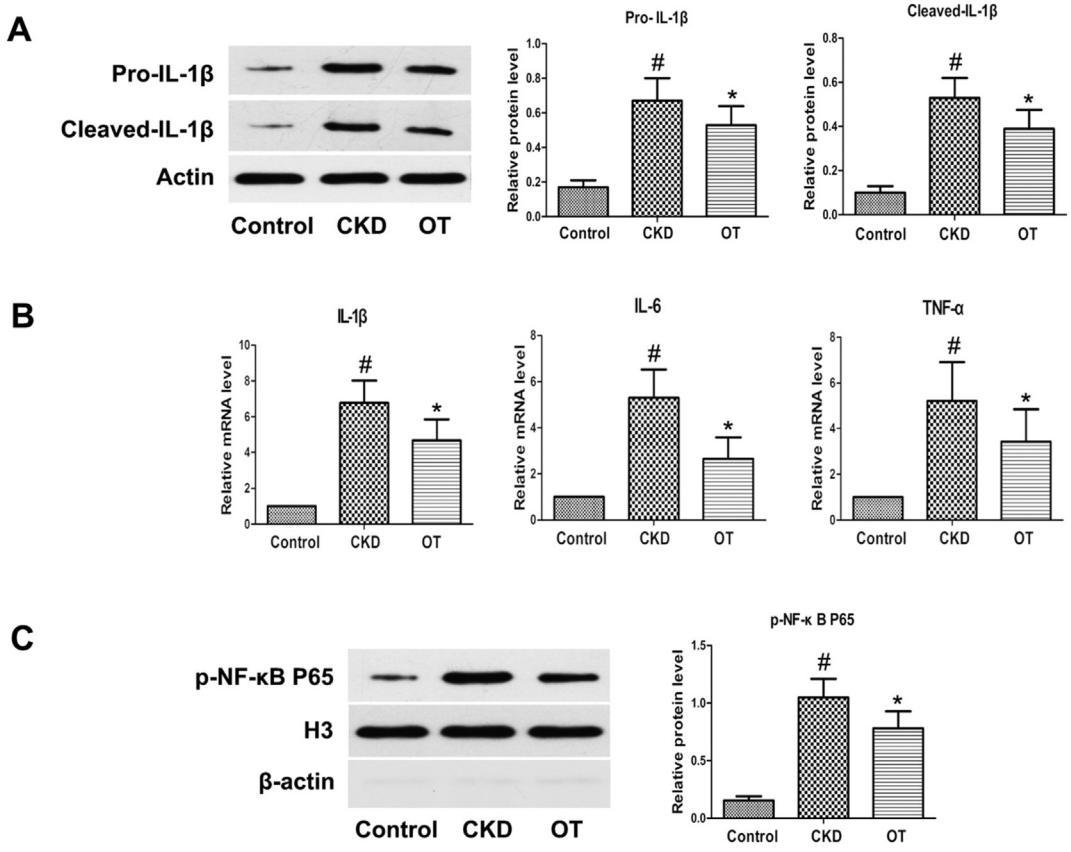

Fig. 3. Effects of ozone therapy on renal inflammation in experimental rats. (A) Representative Western blot images and related quantification bar graphs of pro-IL-1β, cleaved IL-1β and actin. (B) Quantification of mRNA levels of IL-1β, IL-6 and TNF-α. (C) Representative Western blot images and related quantification bar graphs of p-NF-κB P65, H3 and β-actin. Each value is expressed as mean ± SD ($n = 10$). $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs CKD group.

function and serious renal morphological injury, which implied the successful establishment of a CKD rat model.

Calung [29] first revealed that ozone therapy could exert a reno-protective effect to slow down the progression of CKD in 5/6 nephrectomized rats, and its mechanism was associated with the modulation of the renal anti-oxidative system. In accordance with his research, the present study revealed that ozone therapy by rectal insufflation improved renal function and reduced morphology injury in 5/6 nephrectomized rats. This study also revealed that ozone therapy decreased the overexpression of NLRP3, ASC, and caspase-1-p10, together with the reduction of IL-1β as well as other inflammatory cytokines in the kidney of 5/6 nephrectomized rats. These data might indicate a new mechanism whereby ozone therapy could exert its reno-protective effect on 5/6 nephrectomized rats.

Dysregulation of the NLRP3 inflammasome contributes greatly to the chronic renal inflammation in 5/6 nephrectomized rats [30,31]. In this study we investigated the expression of NLRP3, ASC and caspase-1-p10 in the kidney of 5/6 nephrectomized rats. Our results showed that NLRP3, ASC, and cleaved caspase-1 expression levels were significantly upregulated 10 weeks after the previous surgery, while ozone therapy significantly reduced these levels in 5/6 nephrectomized rats, and showed an improvement in renal morphological changes. For the

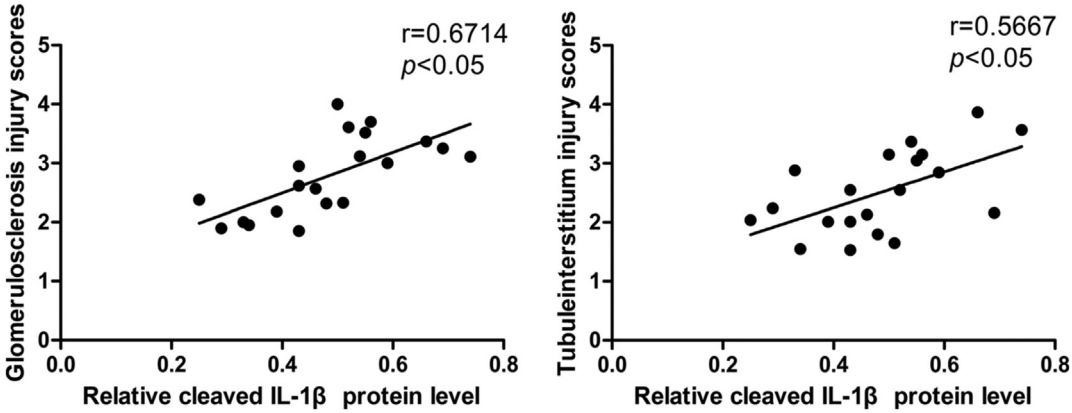

Fig. 4. The relationships between cleaved IL-1β level and renal injury score in kidney of the CKD group and OT group. Each value is expressed as mean ± SD ($n = 20$).

first time, these data indicated that ozone therapy might reduce renal injury through the suppression of the activity of the NLRP3 inflammasome in the kidney of 5/6 nephrectomized rats.

Reducing reactive oxygen species (ROS) or suppressing NF-κB activation was demonstrated to down-regulate NLRP3 inflammasome activity by inhibiting the priming synthesis of NLRP3 protein essential for inflammasome assembly in various diseases [6,32–34]. Ozone therapy is a potent ROS-scavenging treatment that improves the antioxidant system and induces oxidative-stress adaptation in many diseases including CKD [16,17,29]. This evidence indicated that the decreased NLRP3 inflammasome expression in the kidney of 5/6 nephrectomized rats might result from the anti-oxidative property of medical ozone in the present study. Recent research has also suggested that the anti-oxidative property of ozone therapy was achieved by activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [35,36], which has been identified as the main defense against oxidative stress in organs [37]. Interestingly, previous studies revealed that activating Nrf2 could negatively regulate NLRP3 inflammasome expression via its downstream anti-oxidative products NQO-1 and HO-1, both of which were effective ROS scavengers [38,39]. According to these pieces of evidence, we hypothesized that the downregulated expression of the NLRP3 inflammasome in 5/6 nephrectomized rats receiving ozone therapy in the present study might be attributed to the ROS-scavenging property of medical ozone via the modulation of Nrf2.

Alternatively, ozone treatment might downregulate the expression of the NLRP3 inflammasome by its NF-κB-suppressing activity which was also observed in the present study [13,40]. However, the latest research has reported that the activity of NF-κB might be suppressed by the Nrf2/Keap1 pathway in various diseases, both in direct and indirect ways [41,42]. As a result, perhaps the regulation of Nrf2 might also be involved in down-regulating NLRP3 inflammasome expression through the NF-κB-suppressing activity of ozone therapy. However, more studies are needed to elaborate on this issue.

This study also investigated some important inflammatory cytokines including IL-1β, IL-6 and TNF-α in the kidney of 5/6 nephrectomized rats. The cytokine IL-1β is considered as a central player in persistent renal inflammation in CKD [43]. Moreover, it leads to dysfunction of renal tubular epithelia through the suppression of the activation of the renal tubular potassium channel and also enhances the expression of extracellular matrix enzymes in tissue [44,45]. In this study, ozone therapy simultaneously decreased pro-IL-1β and cleaved IL-1β expression in 5/6 nephrectomized rats. These results indicated that ozone therapy might reduce renal injury by decreasing IL-1β infiltration and inhibiting IL-1β activity in 5/6 nephrectomized rats. The reduction of pro-IL-1β might be explained by the NF-κB-suppressing effect of ozone therapy which was observed in previous studies [13,40] as well as the present one. Ozone therapy also decreased IL-6 and TNF-α levels in these rats, which might partly result from the downregulation of cleaved IL-1β, a crucial regulator recruiting IL-6 and TNF-α [46], suggesting the anti-inflammatory effect of ozone therapy in 5/6 nephrectomized rats.

In the canonical way, the activation of leukocyte-associated NLRP3 inflammasome could increase cleaved IL-1β levels to aggravate renal inflammation [7]. In this study, the downregulated activity of the NLRP3 inflammasome and the reduction of inflammatory cytokines centered on cleaved IL-1β were simultaneously observed in the kidney of 5/6 nephrectomized rats receiving ozone therapy. Moreover, the results showed that the protein level of IL-1β was positively correlated with renal injury scores including glomerulosclerosis and tubulointerstitial injury scores, which further implied that NLRP3 inflammasome activation is a driving mechanism of kidney injury in 5/6 nephrectomized rats. Taken together, these results showed that ozone therapy might alleviate renal inflammation by modulating the NLRP3 inflammasome in the kidney of 5/6 nephrectomized rats. In addition, this conclusion might explain the suppression of IL-1β by ozone therapy in many diseases through a new mechanism [47]. However, some evidence on the non-canonical pathways suggested that the activation of renal-associated NLRP3 caused renal tubular mitochondrial dysfunction and led to tubulointerstitial injury [48,49]. Bakker revealed that the activation of renal-associated NLRP3 impaired tubular epithelial response and increased the incidence of IR-induced CKD in NLRP3-deficient mice [50]. Both renal tubular mitochondrial dysfunction and insufficiency of tubular epithelial response could induce sterile inflammation by upregulating IL-1β, IL-6, and TNF-α levels in the kidney. Therefore, it is difficult to determine whether the reno-protective action of ozone therapy is achieved by the modulation of renal-associated NLRP3 or leukocyte-associated NLRP3, or both. Moreover, whether ozone treatment exerts its inhibiting effect on the NLRP3 inflammasome through the expression of other molecules in the complex immune system was not comprehensively investigated in this study. Finally, long-term observations at different time points, in the study of CKD, were not evaluated in this study. Further studies are needed to elucidate these issues.

5. Conclusion

This study first suggested that the reno-protective effect of ozone therapy might be achieved by its anti-inflammatory property through the modulation of the NLRP3 inflammasome in 5/6 nephrectomized rats. Possibly, regulating the NLRP3 inflammasome might account for the inflammation-suppressing effect of ozone therapy in other diseases. Our study, together with some previous ones [29,51], indicates that ozone therapy, as a promising but neglected treatment, might be a beneficial and cost-effective medical strategy for restoring renal function of patients with CKD.

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