Ozone therapy could attenuate tubulointerstitial injury in adenine-induced CKD rats by mediating Nrf2 and NF-κB

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Introduction

Chronic kidney disease (CKD), an irreversible disease that results in end-stage renal failure (1), has been a substantial health concern for many years. According to the recent Global Burden of Disease study, CKD ranked 27th in 1990 but increased to 18th in 2010 in the list of the leading causes of death worldwide (2); as such, the economic burden caused by CKD has increased. Thus far, except expensive renal replacement therapy, only few effective therapy agents have been developed for treatment of CKD in recent years (3). Therefore, new therapy agents especially those cost-effective medical treatments are required by international medical community (3, 4).

Irrespective of primary diseases, chronic tubulointerstitial injury is a common pathway aggregating CKD (5, 6). Studies revealed that impaired Nrf2 activation, which results in diseased anti-oxidative system and oxidation-mediated injury, is one of critical causes that lead to chronic tubulointerstitial injury in CKD(7, 8). As the main guard defending oxidative stress injury in organs, Nrf2 generally forms a multimer with Kelch-like ECH-associated protein1 (Keap1) to maintain an inactive state, but Nrf2 would release from the multimer and bind to the sites of antioxidant response element (ARE) or electrophile-responsive element (EpRE) in nucleus when cells are exposed to oxidative stress. This condition, in turn, mobilizes antioxidant enzymes and if phase detoxification enzyme to resist insults (9). In addition, promoting Nrf2 activation also suppresses inflammation by negatively mediating nuclear factor-kappa B (NF-κB) signaling pathway in some diseases (10). Therefore, treatment involving restoring the impaired Nrf2 system could be a strategy to alleviate tubulointerstitial injury induced by oxidative stress and inflammation in CKD.

Ozone is a toxic gas but could be a safe therapeutic agent if applied in suitable amounts (11). Ozone therapy has been widely accepted by medical community as a safe and cost-effective treatment for...
many diseases such as organ ischemia reperfusion injury and skin ulcer (12-15). Lately, some researches proved ozone therapy could activate Nrf2 of peripheral blood mononuclear cells and human endothelial cells in healthy human to defend insults (16, 17). However, no study has investigated if ozone therapy could attenuate chronic tubulointerstitial injury in CKD by regulating impaired Nrf2 activation. Hence, this study aims to investigate this subject by creating a rodent model of CKD induced by adenine diet.

Materials and Methods

Animals and experimental design

Male Sprague-Dawley rats weighting about 180-200 g (8 weeks of age) were obtained from Animal Experimental Center of Tongji Medical College, Wuhan, China. The rats were housed in a room with a stable temperature of 22-23 °C, humidity at 50%-60% and a 12:12-hr light-dark cycle. The rats were fed with a standard diet and water ad libitum. All the experiment procedures were approved by the Experiment Animal Center Committee in Wuhan University, and the procedure was performed in accordance with the international animal care guidelines.

According to previous researches (15, 18, 19), the rats were fed with 0.75% adenine diet for 4 weeks to induce tubulointerstitial injury and irreversible CKD. As previous researches described (13, 20), ozone therapy was conducted with YKS-1000 g ozone therapeutic instrument (Ikou Co, Ltd, Zhuhai, China) to generate 3% ozone/oxygen gas mixture. The ozone concentration was set at 50 μg/ml. After determining the body weight of rats, we set the therapeutic dose to 1.1 mg/kg, which is proved to be safe and effective referring to the previous research (21). The gas mixture was administrated through rectal insufflation using a polyethylene cannula.

After 7 days of acclimation, the rats were divided into 3 groups: 1) Sham group: rats were fed with normal diet only. 2) ADE group: rats were fed with 0.75% adenine diet and were not subjected to any treatment. 3) OT group: rats were fed with the same diet as ADE group, but simultaneously administrated with ozone therapy. The experiment was terminated after 4 weeks, all the rats were sacrificed 24 hr after ozone therapy. Blood and kidney samples were collected for further analysis.

Biochemical analysis

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

Kidney tissue was sequentially rinsed with PBS (0.01 M, pH=7.4), weighted and homogenized in PBS. Then the homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. After that, the supernatant was collected to detect SOD, CAT, GSH, MDA, PCO concentration by using corresponding commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer instruction. Absorbance of SOD, CAT and GSH was respectively measured at 450 nm, 405 nm and 450 nm, while absorbance of MDA and PCO was respectively determined at 532 nm and 370 nm by using a spectrometer. Results were normalized to the total protein concentrations of renal tissue homogenate samples measured with Coomassie blue method by using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Pathological analysis

The kidney was embedded with paraffin after 24 hr of paraformaldehyde fixation and sectioned to slides at 3 μm thick. The slides were subjected to hematoxylin–eosin (H&E) staining and Masson trichrome (MT) staining and then assessed under a microscope.

Immunohistochemical staining was performed. The slides were deparaffinized in xylene and rehydrated with ethanol gradient washes. Hydrogen peroxide (3%) was applied to activate endogenous hydrogen peroxide. The slides were sequentially retrieved using antigen microwave retrieval technique in a citrate buffer solution and blocked with 10% normal goat serum. The slides were incubated with primary antibodies against Nrf2 (ab31163, Abcam, Cambridge, MA, USA) and p-NF-κB P65 (sc-33020, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After washing three 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.

Western blot analysis

Cytoplasmic and nuclear proteins were extracted from the frozen kidney by using OP-0002 and OP-0006 EpiQuik™ protein extraction kit (Epigentek, Farmingdale, NY, USA), respectively, according to the manufacturer’s instruction. Protein concentration was determined using a Bradford protein assay reagent kit (Bio-Rad, Richmond, CA, USA) according to the manufacturer’s instruction. The extracted proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and blocked with 5% non-fat milk in TBST buffer. The membranes were incubated with
Trichrome staining was performed in the kidneys of different groups, original magnification ×400 (A); Masson trichrome staining was performed in the kidneys of different groups, original magnification ×200 (B). Sham: normal control group; OT: ozone therapy group; ADE: adenine-induced CKD group.

Figure 1. Effect of ozone therapy on tubule-interstitial morphology at week 4. Hematoxylin and eosin staining was performed in the kidneys of different groups, original magnification ×400 (A); Masson trichrome staining was performed in the kidneys of different groups, original magnification ×200 (B).

Table 1. Primer list

| Gene   | Sequence                                      | Length (bp) |
|--------|-----------------------------------------------|-------------|
| IL-1β  | Forward: 5′-CTCGACTGGTGATGATG-3′ Reverse: 5′-AGGAGTGTGTGGTCTGTG-3′ | 211 bp      |
| IL-6   | Forward: 5′-AGTGCTTCTTGGGACTGA-3′ Reverse: 5′-ACAGTCTGATATGCCGCTTTC-3′ | 218 bp      |
| TNF-α  | Forward: 5′-GGGCCCTTCACTCGAGGTTT-3′ Reverse: 5′-CCCGCTTAATTGGTAAATGAC-3′ | 180 bp      |
| ICAM-1 | Forward: 5′-TGTCAAGGGGAGATGATTGC-3′ Reverse: 5′-GGCGGGAATAGGTGTAAATGGAC-3′ | 185 bp      |
| GAPDH  | Forward: 5′-ACACGAAGGGATGGTGGGAC-3′ Reverse: 5′-TTRAGGTGTCAGCGAATCT-3′ | 253 bp      |

cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, extension at 72 °C for 1 min; and final extension at 72 °C for 7 min. Data were presented as the ratio of genes to GAPDH mRNA and assessed with GraphPad Prism 5 software.

Statistics analysis

SPSS 17.0 was used for statistics analysis. Date was presented as mean±SEM. One-way ANOVA 3 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 nuclear expression of Nrf2 and IL-1β in experiment rats. P<0.05 was considered as statistically significance.

Results

Effect of ozone therapy on renal function in experimental rats

Renal function was reflected by serum Cr, BUN, K, Ca and IP concentration. Compared with Sham group, Cr, BUN, K, Ca and IP level were all significantly higher in ADE group (P<0.01; Table 2). OT group showed a significant decrease in these indexes than ADE group (P<0.05; Table 2).
Table 2. Serum biochemical parameters in different groups at week 4

| Serum biochemical indexes | Sham group | OT group | ADE group |
|---------------------------|------------|----------|-----------|
| Cr (um/l)                 | 30.33±3.88 | 128.22±14.41* | 221.33±19.13# |
| BUN (um/l)                | 3.99±0.42  | 35.31±5.16* | 64.03±7.70# |
| K (mmol/l)                | 4.71±0.21  | 5.35±0.34* | 6.41±0.46# |
| Ca (mmol/l)               | 2.31±0.20  | 2.58±0.18* | 2.81±0.18# |
| IP (mM/l)                 | 2.53±0.27  | 4.41±0.28* | 4.79±0.36# |

Date was represented by mean±SEM. * P<0.05, versus ADE group; # P<0.01, versus Sham group. Sham group: normal control group; OT group: ozone therapy group; ADE group: adenine-induced CKD group.

Effect of ozone therapy on tubule-interstitial pathology

H&E staining and MT staining were performed to detect tubule-interstitial pathological changes in experiment rats. Compared with Sham group, rats in ADE group developed more serious tubule-interstitial injury, as evidenced by tubular expansion, loss of brush border of proximal tubules, tubular atrophy, serious inflammatory cells infiltration and terrible tubule-interstitial fibrosis. However, no remarkable glomerular injury was observed (Figure 1). Changes mentioned above were mainly observed in the kidney cortex. However, compared with ADE group, tubule-interstitial injury significantly improved in OT group (Figure 1).

Effect of ozone therapy on anti-oxidation enzymes and oxidation productions in the kidneys of experimental rats

Some typical anti-oxidation enzymes and oxidation productions were detected to reflect changes in anti-oxidative and oxidative system in the kidneys of experimental rats. The level of anti-oxidation enzymes including SOD, CAT and GSH were significantly lower in ADE group than Sham group (P<0.01; Table 3), while the levels of oxidative productions including MDA and PCO were significantly higher than Sham group (P<0.01; Table 3). OT group showed a significant increase of SOD, CAT and GSH and a remarkable decrease of MDA and PCO as compared with ADE group (P<0.05; Table 3).

Effect of ozone therapy on Nrf2 in the kidney of experimental rats

The Nrf2 activation in the tubule-interstitium of experiment rat was detected. Immunohistochemistry showed Nrf2 activation in tubule-interstitium area was significantly suppressed in ADE group than Sham group, while the expression of Nrf2 in OT group was significantly increased when compared with ADE group (Figure 2A). Western blot results showed the nuclear protein expression of Nrf2 was remarkably down-regulated in ADE group than Sham group (P<0.01; Figure 2B), while the nuclear protein levels of Nrf2 was significantly up-regulated in OT group than ADE group (P<0.05; Figure 2B).

Effect of ozone therapy on NF-κB-mediated inflammation in the kidneys of experimental rats

To determine the activation of NF-κB signaling pathway in the kidney of experiment rat, the expression of p-IκBα and nuclear p-NF-κB P65 were detected in this paper. Immunohistochemistry results showed p-NF-κB P65 was excessively expressed in the kidney of ADE group than in Sham group, mainly located in the nucleus of kidney tubule epithelial cell (Figure 3A). However, the expression of p-NF-κB P65 in tubule-interstitium of OT group was significantly decreased when compared with that of ADE group (Figure 3A). Western blot results showed the protein expression of cytoplasm p-IκBα and nucleus p-NF-κB P65 was remarkably increased in ADE group than normal control group.
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Table 3. Renal concentration of biochemical parameters in different groups at week 4

| Groups | SOD (U/mgprot) | CAT (U/mgprot) | GSH (μmol/mgprot) | PCO (nmol/mgprot) | MDA (nmol/mgprot) |
|--------|----------------|----------------|--------------------|-------------------|-------------------|
| Sham   | 4.53±0.36      | 95.93±6.95     | 15.27±2.05         | 2.59±0.68         | 0.77±0.10         |
| OT     | 1.26±0.15 *    | 17.35±1.36 *   | 9.13±1.68 *        | 8.07±1.33 *       | 1.80±0.09 *       |
| ADE    | 0.83±0.16 #    | 8.87±0.86 #    | 6.22±1.02 #        | 19.15±3.12 #      | 2.29±0.21 #       |

Date was represented by mean±SEM. * P<0.05, versus ADE group; # P<0.01, versus Sham group. Sham: normal control group; OT: ozone therapy group; ADE: adenine-induced CKD group.

Sham group (P<0.01; Figure 3B), while the levels of these proteins were significantly decreased in OT group than ADE group (P<0.05; Figure 3B).

Some typical inflammatory cytokines were also detected to reflect inflammation infiltration in the kidneys of experimental rats. The protein and mRNA levels of IL-1β, IL-6, TNF-α and ICAM-1 were significantly higher in ADE group than in Sham group (P<0.01; Figure 3C). OT group showed a significant decrease of these inflammatory cytokines levels as compared with ADE group (P<0.05; Figure 3C). The correlation between the nuclear expression of Nrf2 and p-NF-κB P65 in kidney:

As shown in Figure 4, the nuclear protein expression of Nrf2 was negatively correlated with the nucleus protein expression levels of p-NF-κB P65 in the kidneys of experiment rats.

Figure 3. Effect of ozone therapy on NF-κB-mediated inflammation in the kidneys of different groups at week 4. P-NF-κB P65 expression in the tubule interstitium was detected by immunohistochemical staining (A). The protein level of p-IκBα and p-NF-κB P65 in the kidney was detected by western blot analysis (B). The protein level and mRNA level of TNF-α, IL-1β, IL-6 and ICAM-1 were respectively detected by Western blot analysis (C) and real-time PCR analysis (C). Date was represented by mean±SEM. *P<0.05, versus ADE group; **P<0.01, versus ADE group; # P<0.01, versus Sham group. Sham: normal control group; OT: ozone therapy group; ADE: adenine-induced CKD group.
A rat model of adenine-induced CKD was first established by Yokozawa and developed by many scientists. Previous studies reported that persistent 0.75% adenine-containing diet could lead to gradual accumulation of 2,8-dihydroxyadenine in renal tubules, which could induce chronic oxidative stress and inflammation; these phenomena could result in progressive deterioration of renal function and typical chronic tubulointerstitial injury which are similar to clinical patients with CKD(18, 19, 22). In this study, we developed a rat model of CKD induced by adenine and determined the efficacy of ozone therapy for alleviating chronic tubulointerstitial injury. The results of serum biochemical analysis showed that adenine-fed rats suffered severe renal insufficiency and electrolyte homeostasis disorder after a 4 week modeling period; hence, a rat model of severe CKD was successfully established. Furthermore, H&E staining and MT staining analyses indicated that the rats developed severe tubulointerstitial injury. A low dose of ozone therapy by rectal insufflation improved serum biochemical indices and alleviated renal tubulointerstitial injury in rats. These results indicated that long-term ozone therapy at low-dose rectal insufflation may exert a renoprotective action by alleviating tubulointerstitial injury in rats with adenine-induced CKD.

Excessive oxidative stress caused by functional decline in the anti-oxidative system is a crucial factor that causes tubulointerstitial injury in rats with adenine-induced CKD (23, 24). In this study, the biochemical analysis on anti-oxidation enzymes and oxidation production indicated that persistent adenine diet disturbed redox homeostasis and caused serious oxidative stress injury in the kidney of experimental rats. However, ozone therapy reversed this condition by increasing the levels of SOD, CAT, and GSH, which are main defense enzymes against oxygen radical (25, 26); the therapy also decreased the levels of MDA and PCO, which are typical oxidation products (27), in the kidneys of rats with adenine-induced CKD. These results were consistent with other researchers' results (28-30), who demonstrated that ozone therapy could increase the concentrations of anti-oxidative enzymes in other animal disease models. And based on these results, we considered that ozone therapy could enhance the activity of anti-oxidation system and ameliorate oxidative-stress injury oxidative-stress injury in the tubulointerstitium of rats with adenine-induced CKD.

This research is the first to study the effects of ozone therapy on impaired Nrf2 activation, which is considered the main factor that causes functional decline in anti-oxidant system in the tubulointerstitium of rats with adenine-induced CKD (7). As showed in Figure 2, Nrf2 expression in the tubulointerstitium of rats with adenine-induced CKD was suppressed, but ozone therapy up-regulated this expression. These results are consistent with the findings other, who reported that ozone therapy could improve Nrf2 activation in normal peripheral blood cells and human endothelial cells to resist insults (16, 17). According to their studies, ozone therapy could react with polyunsaturated fatty acid to generate H₂O₂ and alkenals including 4-hydroxynonenal, which in turn alters the cysteine residues of Keap1 to inhibit ubiquitin conjugation to Nrf2 by the Keap1 complex and stimulate the release and nuclear translocation of Nrf2 (16, 31). Then the activated Nrf2 could induce the production of anti-oxidative enzymes such as SOD, CAT and GSH by binding to ARE in nucleus. As the health guardians, these free radical scavenging enzymes could convert peroxides including hydrogen peroxide and reactive oxygen radicals to harmless substances such as water and oxygen, eventually decrease lipid peroxidation products such as MDA and ameliorates oxidative stress injury(25, 32). Hence, for the first time, we considered that ozone therapy may improve anti-oxidation activity by restoring impaired Nrf2 in the tubulointerstitium of rats with adenine-induced CKD.

Previous studies confirmed that chronic inflammation mediated by overactivation of IκBα/NF-κB pathway plays a pivotal role in aggravating chronic tubulointerstitial injury in rats with adenine-induced CKD (22, 24). Ozone therapy could reduce inflammation injury in some animal disease models (33, 34). In addition, Leon et al reported that the anti-inflammation effect of ozone therapy was obtained by inhibiting NF-κB activation (20). In the present study, ozone therapy restrained the overactivation of p-IκBα and p-NF-κB P65 in the tubulointerstitium of rats with adenine-induced CKD; the therapy also reduced the levels of inflammatory cytokines, such as ICAM-1, TNF-α, IL-1β, and IL-6,
which were the downstream production of activation of IkBa/NF-kB pathway (35, 36), in the kidneys of these rats. These results were consistent with our previous study (37) and indicated that ozone therapy may attenuate tubulointerstitial inflammation by inhibiting NF-kB signaling pathway in rats with adenine-induced CKD.

The interaction between Nrf2 and IkBa/NF-kB pathway has gained increased research attention (38, 39). Sun et al revealed that activating Nrf22 could inhibit the NF-kB signaling pathway to attenuate muscle inflammation in dystrophin-deficient mdx mice (40). Further studies on lupus nephritis and neutrophilic lung inflammation through gene knockout technology revealed that Nrf2−/− rodents suffered more severe NF-kB-induced inflammation injury than the healthy controls (10, 41). According to these researches, in addition to antioxidant activity, Nrf2 also exerts anti-inflammation activity by directly down-regulating the activation of the IkBa/NF-kB pathway or reducing oxidative stress, which could activate NF-kB. In this study, ozone therapy simultaneously restored impaired Nrf2 activation and suppressed NF-kB-mediated inflammation in the tubulointerstitium of rats with adenine-induced CKD. Moreover, as showed in Figure 4, a negative relationship existed between the nuclear protein level of Nrf2 and p-NF-kB P65. Based on these results, the anti-inflammation property of ozone therapy may be partly achieved by restoring impaired Nrf2 activation in the tubulointerstitium of rats with adenine-induced CKD. However, the present evidence was insufficient to verify this assumption. Moreover, the present study did not evaluate the long-term effect of ozone therapy and consider different therapy concentrations and detection time points. Thus, further studies are needed to confirm and reveal the specific mechanisms involved in therapy for CKD.

**Conclusion**

This study is the first to report that ozone therapy could improve chronic tubulointerstitial injury and exert renoprotection effect by restoring impaired Nrf2 activation and down-regulating NF-kB activation in rats with adenine-induced CKD. Based on this evidence and our previous study, we speculated that ozone therapy, which is a low-cost and effective non-drug treatment that has been clinically use for many years, may be a potential medical strategy to slow down disease progression in patients with CKD by alleviating chronic tubulointerstitial injury. However, further animal and clinical studies must be performed to confirm the results.

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**Conflict of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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