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ORIGINAL ARTICLE

Ozone therapy prevents renal inflammation and fibrosis in a rat model of acute pyelonephritis

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Abstract

Introduction. Not only bacterial characteristics but also oxidative/nitrosative stress could play a significant role in renal parenchymal inflammatory processes in acute pyelonephritis (APN). This study was conducted to evaluate the effect of ozone therapy (OT), as an immunomodulator and antioxidant, on the renal function, morphology and biochemical parameters of oxidative stress in an experimental model of APN in rats. Materials and methods. Forty rats were divided equally into five groups as control, APN, APN + Antibiotic, APN + OT, and APN + Antibiotic + OT. APN was induced by 0.1 ml of freshly prepared Escherichia coli (ATCC 25922) solution containing 10¹⁰ colony-forming unit/ml into the kidney. A control group was administered 0.1 ml of 0.9 % NaCl solution. Treatment was begun 72 h after bacterial inoculation. Control and APN groups were given 0.9% NaCl solution, APN + Antibiotic and APN + OT were given either antibiotic (ciprofloxacin 150 mg/kg intramuscular/twice daily) or OT. APN + Antibiotic + OT group was given both antibiotic and OT for five consecutive days. At the end of the seventh day, animals were killed via decapitation and trunk blood was collected. Both kidneys were harvested and one half of each kidney were immediately stored for antioxidant enzyme activity, tissue lipid peroxidation and protein carbonyl content. The remainder was fixed for histopathologic examination. Results. E. coli-induced APN increased the renal glomerular and tubular dysfunction, oxidative stress parameters and antioxidant enzyme activities. Either antibiotherapy or OT markedly ameliorated renal dysfunction, the antioxidant status of the kidneys and histopathological injuries subjected to E. coli-induced APN. Interestingly, the combination of antibiotherapy and OT was much more effective than either of the treatment modalities alone. Conclusion. The combination of antibiotherapy and OT markedly ameliorated renal dysfunction and improved antioxidant status and histopathologic modalities in rats subjected to E. coli-induced APN than either antibiotherapy or OT treatment alone. Therefore, OT may be considered as an adjuvant therapy to classical antibiotherapy to prevent renal inflammation and fibrosis in APN.

Key Words: Kidney, pyelonephritis, fibrosis, ozone, oxidative stress

Introduction

Urinary tract infections are the most common serious bacterial infections among young children with acute pyelonephritis (APN) and APN-associated renal scarring. This phenomenon causes the most potential long-term damage to a child's health [1]. Acute pyelonephritis is still one of the most common serious infections in infancy and childhood, and renal scars following APN is the major cause of chronic renal failure [1,2]. The formation of scars following APN has been assumed to depend on bacterial characteristics and inflammatory reactions [3]. This process is mainly proportional to the extent of inflammation associated with infection, rather than actual bacterial growth in the kidney [2]. Bacterial endotoxin is responsible for initiating the inflammatory response and cytokine cascade in monocytes/macrophages which result in the secretion of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-8), while renal tubular epithelial cells also secrete chemokines, and cytokines [4]. The consequence of chemokine/cytokine secretion is to establish chemotactic gradients to attract mononuclear cells and polymorphonuclear cells (PMNs) to the site of infection.
Phagocytosis of bacteria is commenced with recruitments of activated PMNs, monocytes and mesangial cells to inflammatory areas [2,6,7]. Phagocytosis triggers oxidative or respiratory burst in PMNs and neutrophils, which results in a sequential production of reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, and reactive oxygen species (ROS), such as superoxide ion, hydrogen peroxide, hypochlorous acid and various other species [7]. Large amounts of these free radicals may contribute to the tissue damage causing the formation of scars in the kidney. Reports suggest that ROS and RNS play an important role in the pathophysiology of APN [2,3,6,8]. Taken together, it should be pointed out that not only bacterial characteristics but also oxidative/nitrosative stress could play a significant role in renal parenchymal inflammatory process and progressive kidney damage.

A gas mixture comprising ozone (O\textsubscript{3})/oxygen (O\textsubscript{2}) has long been known as medical ozone therapy (OT). An ozone/oxygen mixture exhibits various effects on the immune system, such as the modulation of phagocytic activity on peritoneal and alveolar macrophages that generate the first defense against bacteria and their toxins [9–11]. The utilization of O\textsubscript{3} as a potent antimicrobial agent has been the object of many studies. It was shown that bacteria, spores, and viruses are inactivated by O\textsubscript{3} after only a few minutes of exposure [10]. Clinical studies have so far shown that OT appears useful in septic diseases including peritonitis, infected wounds, chronic skin ulcers, initial gangrene, burns and advanced ischemic diseases [11–14]. It has also been reported that administration of ozone induces a sort of cross-tolerance to free radicals released after hepatic and renal ischemia-reperfusion in experimental studies [15,16]. We recently reported the ameliorative effects of OT on oxidative stress in several experimental models such as acute necrotizing pancreatitis [17], caustic esophageal burn [18] and necrotizing enterocolitis [19].

Taking into account that OT has therapeutic properties as an antimicrobial and modulator of antioxidant defense system, this study was conducted to evaluate the effects of OT on the renal function, morphology and oxidative stress parameters of an experimental model of APN in rats.

**Materials and methods**

**Animals**

Sprague-Dawley male rats (200–250 g) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature and relative humidity were kept constant. Rats were fed with standard rat chow and tap water ad libitum. All experimental protocols were approved by a local Animal Care and Use Committee.

**Experimental groups and surgery**

Forty rats were divided equally into five groups; control, APN, APN + AB, APN + OT, APN + AB + OT. Following a 12 h fasting period, animals were anesthetized with an intraperitoneal (ip) injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg). Both kidneys of rats were exposed through an upper midline incision and 0.1 ml of a freshly prepared *Escherichia coli* (ATCC 25922) solution containing $10^{10}$ colony-forming units/ml were injected into their kidneys through the cortex to the medulla. The control group was administered 0.1 ml of 0.9% NaCl solution. Finally, the abdominal incision was closed, and the animals were returned to their cages to recover where water and food were available ad libitum. To investigate the acute inflammatory process and oxidative stress status that occur in response to bacterial multiplication of the kidney parenchyma, the animals were killed via decapitation at the end of the seventh day and trunk blood was collected and centrifuged to study biochemical parameters in serum. Both kidneys were harvested immediately and bisected sagittally. One half of each kidney was immediately stored at $-80 \, ^\circ \text{C}$ for antioxidant enzyme activity and tissue lipid peroxidation levels, the remainder was fixed in 10% formaldehyde for histopathologic examination.

**Treatment modalities**

Treatment was begun 72 h after bacterial inoculation. Control and APN groups were given 1 ml/kg of 0.9% NaCl solution via ip injection daily. APN + AB and APN + AB + OT were given antibiotic (ciprofloxacin 150 mg/kg intramuscular) twice daily for 5 days. The rats in the APN + OT and APN + AB + OT groups were administered an ozone/oxygen mixture at a dose of 0.7 mg/kg daily via the ip route for five consecutive days.

Medical ozone was generated by the ozone generator (OZONOSAN Photonik 1014, Hansler GmbH, Nordring 8, Ilfezheim, Germany), allowing control of the gas flow rate and ozone concentration in real time by a built-in UV spectrometer. The ozone flow-rate was kept constant at 3 L/min representing a concentration of 60 mg/ml and gas mixture of 97% O\textsubscript{2} + 3% O\textsubscript{3}. Tygon polymer tubes and single-use silicon-treated polypropylene syringes (ozone resistant) were used throughout the reaction to ensure containment of O\textsubscript{3} and consistency of concentrations.

**Tissue preparation and biochemical analysis**

The frozen tissues were homogenized in phosphate buffer (pH 7.4) by means of a homogenizer (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany) on an ice cube. The supernatant
was used for the entire assay. Initially, the protein content of tissue homogenates was measured by the method of Lowry [20] with bovine serum albumin as the standard used for all assays.

Lipid peroxidation level was measured with the thiobarbituric acid (TBA) reaction by the method of Ohkawa [21]. This method was used to obtain a spectrophotometric measurement (Helios, Epsilon, USA) of the color produced during the reaction to thiobarbituric acid (TBA) with malondialdehyde (MDA) at 535 nm. The calculated MDA levels were expressed as mmol/g-protein.

Tissue protein carbonyl content (PCC) was determined spectrophotometrically by the method based on the reaction of the carbonyl group with 2, 4-dinitrophenylhydrazine to form 2, 4-dinitrophenylhydrazone [22]. 2, 4-Dinitrophenylhydrazine was the reagent originally used for proteins subjected to metal-catalysed oxidation. Absorbances were measured with a spectrophotometer. The results were given as millimoles (mmol) carbonyl per gram protein.

Superoxide dismutase (SOD) activity was assayed using the nitroblue tetrazolium (NBT) method of Sun et al. and modified by Durak et al. [23]. In this method, NBT was reduced to blue formazan by O$_2^-$, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The estimated SOD activity was expressed as Units per gram protein.

The glutathione peroxidase (GSH-Px) activity was measured using the method described by Paglia and Valentine [24] in which GSH-Px activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37°C. The absorbance at 340 nm was recorded for 5 min. The activity was the slope of the lines as mmol of NADPH oxidized per minute. GSH-Px activity was presented as U/g-protein.

Serum samples were used for the measurement of serum creatinine ($S_{Cr}$) and urea levels, which were used as indicators of impaired glomerular function, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), which was used as an indicator of renal injury [25]. Serum urea, $S_{Cr}$, LDH and AST were determined with an Abbott-Aeroset autoanalyser (Chicago IL, USA) using original kits.

Serum neopterin (NP) levels were determined by using a High Pressure Liquid Chromatography (HPLC) system with a fluorescence detector as prescribed previously [26] and presented as nmol/L.

Serum Procalcitonin (PCT) levels were measured by automated immunofluorescent assays (PCT Sensitive KRYPTOR; Brahms Diagnostica, Berlin, Germany) following the manufacturer’s instructions. The assay has a detection limit of 0.02 ng/mL and a coefficient of variation of 20% at a concentration of 0.06 ng/mL.

### Results

All animals survived until the end of the study. Since the bilateral kidneys were harvested to study the oxidant/antioxidant parameters and give a histopathologic evaluation, the sample number was considered as twice the number of animals for each group.

All serum biochemical values are summarized in Table I. There was a significant rise in the $S_{Cr}$ and urea levels in the APN group suggesting a significant degree of glomerular dysfunction ($p<0.05$; APN vs. the other groups). Both antibiotic and OT reduced the $S_{Cr}$ and urea levels in the rats subjected to the APN procedure ($p<0.05$; treatment groups vs. APN). In addition, serum LDH and AST levels, markers of renal injury, were higher in the APN group than in the others ($p<0.05$; APN vs. the others). However, these values were reduced in the treatment groups suggesting that treatment modalities well-preserved renal units ($p<0.05$; APN vs. treatment groups).

Table II displays the outcome of tissue levels of MDA and PCC. Renal MDA and PCC levels were significantly elevated in the APN group suggesting an increased lipid peroxidation and protein oxidation ($p<0.05$; APN vs. the treatment groups). Although, MDA and PCC levels were lower in the treatment groups than the APN group ($p<0.05$; treatment group vs. APN), this decrease was significantly higher.

### Histopathologic evaluation

Both kidneys of each animal were taken for histopathologic evaluation. In all groups, samples of kidney were placed in 10% formalin and processed through to paraffin. They were subsequently sectioned at 3 μm thick and stained with Hematoxylin & Eosin (H&E) and Masson’s trichrome and examined by light microscopy. The sections were scored with a semiquantitative scale based on general microscopic criteria to evaluate the degree of renal damage (inflammation and interstitial fibrosis). The scoring system for inflammation was 0, absent; 1, mild; 2, moderate; 3, severe/focal; 4, multi-focal, and for interstitial fibrosis it was 0, absent; 1, focal, 2, multifocal. Blind analysis of the histological samples was performed by two independent experts.

### Statistical analysis

All biochemical data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, Version 15.0, Chicago, IL, USA). Differences in measured parameters among the three groups were analysed by Kruskal-Wallis test. Dual comparisons between groups that present significant values were evaluated with the Mann-Whitney U test. Statistical significance was accepted as a value of $p<0.05$. 
Table I. Biochemical evaluation of serum for each groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=8)</th>
<th>APN (n=8)</th>
<th>APN + AB (n=8)</th>
<th>APN + OT (n=8)</th>
<th>APN + AB + OT (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mmol/L)</td>
<td>47.7 ± 1.8</td>
<td>57.5 ± 2.7</td>
<td>49.5 ± 2.1</td>
<td>43.3 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>17.2 ± 1.1</td>
<td>22.4 ± 0.8</td>
<td>18.4 ± 0.7</td>
<td>18.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td></td>
<td>574 ± 6.3</td>
<td>612 ± 4.7</td>
<td>525 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td></td>
<td>157 ± 5.8</td>
<td>113 ± 3.4</td>
<td>113 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>PCT (nmol/L)</td>
<td></td>
<td>30 ± 3.1</td>
<td>40 ± 3.4</td>
<td>35 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Neopterine (nmol/L)</td>
<td></td>
<td>0.01 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
</tbody>
</table>

APN: E.-coli-induced APN
AB: Antibiotics
OT: Ozone

Discussion

The results of our study show that E.-coli-induced APN increases the renal glomerular and tubular dysfunction in rats. In addition, we found that experimental APN in rats causes a rise in oxidative stress parameters/antioxidant enzyme activities, serum level of PCT and neopterine. Histological examination of kidneys with APN in rats displayed diffuse accumulation of inflammatory cells and mild to moderate fibrosis. Either antibiotic therapy or OT markedly ameliorated renal dysfunction, the antioxidant status of kidneys and histopathological injuries subjected to E. coli-induced APN. Interestingly but not surprisingly, the combination of antibiotic therapy and OT was much more effective than either of the treatment modalities alone.

We found that E. coli-induced APN elevated oxidative stress markers (i.e., MDA and PCC) and reduced the antioxidant enzyme activities (i.e., SOD and GSH-Px). The renal inflammatory process following bacterial invasion in APN involves a complex
Table II. Biochemical evaluation of kidneys for each group.

<table>
<thead>
<tr>
<th></th>
<th>MDA (mmol/g-protein)</th>
<th>PCO (mmol/g-protein)</th>
<th>SOD (U/g-protein)</th>
<th>GSH-Px (U/g-protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 16)</td>
<td>0.090 ± 0.007</td>
<td>0.053 ± 0.008</td>
<td>484.9 ± 16.7</td>
<td>9.4 ± 0.8</td>
</tr>
<tr>
<td>APN (n = 16)</td>
<td>0.187 ± 0.011 a, b, c, d, e</td>
<td>0.138 ± 0.010 a, b, c, d, e</td>
<td>305.2 ± 12.3 a, b, c, d, e</td>
<td>5.4 ± 1.2 a, b, c, d, e</td>
</tr>
<tr>
<td>APN + Anb (n = 16)</td>
<td>0.129 ± 0.009 a, b, c, d, e</td>
<td>0.109 ± 0.011 a, b, c, d, e</td>
<td>452.4 ± 11.3 a, b, c, d, e</td>
<td>8.6 ± 0.9 a, b, c, d, e</td>
</tr>
<tr>
<td>APN + OT (n = 16)</td>
<td>0.108 ± 0.008 a, b, c</td>
<td>0.091 ± 0.012 a, b, c</td>
<td>475.8 ± 15.7 a, b, c</td>
<td>9.4 ± 0.8 a, b, c</td>
</tr>
<tr>
<td>APN + Anb + OT (n = 16)</td>
<td>0.089 ± 0.012 a, b, c, d</td>
<td>0.069 ± 0.007 a, b, c, d</td>
<td>517.4 ± 17.4 a, b, c</td>
<td>10.5 ± 1.1 a, b, c, d</td>
</tr>
</tbody>
</table>

*pSignificantly different from control. **pSignificantly different from APN. ***pSignificantly different from APN + Anb. ****pSignificantly different from APN + OT. 5pSignificantly different from APN + Anb + OT.

and interrelated sequence of events resulting in injury to, and the eventual death of, renal cells leaving scar formation [6]. Inflammation within the kidney involves the production and release of biologically active mediators and pro-inflammatory cytokines from migrating inflammatory cells such as PMNs and glomerular mesangial cells [29]. Activated pro-inflammatory PMNs attract to, and infiltrate, renal tissues where they generate ROS/RNS [30]. The accumulation of ROS/RNS leads to damage in cellular components such as peroxidation of polyunsaturated lipids, forming MDA, and oxidation of amino acid residues on proteins forming protein carbonyls. To eliminate toxic ROS/RNS, cells are equipped with various antioxidant defense systems such as SOD, which converts superoxide (O$_2^-$) to H$_2$O$_2$, and GSH-Px, which breaks down H$_2$O$_2$ to H$_2$O and O$_2$. These enzymes and antioxidant systems protect the cell against lipid peroxidation and protein oxidation. Therefore, MDA, PCC, SOD and GSH-Px are frequently used to confirm the involvement of free radicals in cell damage. Our finding displayed that E. coli-induced APN increased production of lipid peroxidation and protein oxidation in kidneys suggesting the involvement of ROS/RNS in this process. Furthermore, elevated antioxidant enzyme activities in insulted kidneys support the notion that renal injury is induced, at least in part, by toxic oxygen metabolites. Several studies have demonstrated that ROS/RNS play a pivotal role in the pathophysiology of pyelonephritis. Gupta et al. clearly showed the role of ROS in the pathogenesis of E. coli-induced APN [6,31]. It was also shown that ROS are generated at the initiation of infection and their levels progressively increased during the course of infection [8]. Pavlova et al. displayed that elevated levels of lipid peroxidation and antioxidant enzyme capacity in combination with clinical data are proper markers for inflammatory and immunological active parenchymal kidney disorders in children [1]. In addition, excessive production of ROS following stimulation of human PMN’s by uropathogenic P. aeruginosa in vitro was reported by Oh-oka et al. [7]. In agreement with these results, several experimental studies have shown that administration of antioxidant agents such as vitamin A, vitamin E, melatonin and pentoxifylline ameliorate renal scarring [32–34]. In view of this evidence, we can conclude that excessive production of ROS/RNS contributes to the development of renal damage in APN. Thus, it is logical to find new treatment modalities to eliminate or decrease the formation of ROS/RNS in APN for prevention of renal damage.

In this study, we used OT as a new treatment modality against E. coli-induced experimental APN. Our study shows that OT decreased oxidative stress markers and increased antioxidant enzyme activities in this experimental APN model. These biochemical processes are also supported by histopathologic injury scores. Together, findings suggest that OT might scavenge ROS/RNS, maintain antioxidant enzyme activities and levels, and repair ROS/RNS-mediated cellular damage.

After administration, ozone is dissolved in biological fluids such as plasma, lymph and urine; it immediately reacts with polyunsaturated fatty acids (PUFA), proteins, carbohydrates and antioxidants [35]. All of these compounds act as electron donors and undergo oxidation, resulting in the formation of hydrogen peroxide (H$_2$O$_2$) and lipid oxidation products (LOPs). H$_2$O$_2$, an essential ROS molecule, is able to act as an ozone messenger for eliciting several biological and therapeutic effects [9,10,36]. The concept that H$_2$O$_2$ is always harmful has been widely revised because, in physiological amounts, it acts as a regulator of signal transduction and represents a crucial mediator of host defense and immune responses [14,35,37]. Thus, the process of ozonation has been characterized by the formation of H$_2$O$_2$ and LOPs mainly acting two phases. H$_2$O$_2$ is the earliest messenger rising immediately and...
disappearing within 1 min (early and short-acting messenger), while LOPs, via the circulation, distribute throughout the tissues and become late and long-lasting messengers [10,35]. This process stimulates the innate immune system and helps the cell to survive. We assume that all this process might generate a number of messengers able to modulate oxidative/antioxidative status which resulted in decreased tissue damage markers such as MDA and PCC in this study. The significant stimulation of endogenous SOD and GSH-Px in the kidneys induced APN and, when treated with OT in comparison with the APN group, suggests cellular protection is most likely through the reduction in the availability of superoxide anion. It has been demonstrated that OT is able to promote an oxidative preconditioning through the increase and preservation of endogenous antioxidant systems involving glutathione, SOD, and catalase and enzymatic reactions which prepare the host to face physiopathological conditions mediated by ROS/RNS [14,15,19,38,39]. Several experimental studies showed that OT therapy ameliorates the tissue injury due to free radicals in various forms of shock, stroke, inflammation, and reperfusion injury [9,10,40–45]. Another possible explanation for the protective effect of OT is that it is a powerful disinfectant. Although the most acceptable theory is that ozone can disrupt the function of the bacterial cell membrane and cell integrity by exerting its strong oxidizing capability, under in vivo circumstances, it may also affect indirectly via stimulating the leukocytes and immune system [9,46,47]. Taken together, we can conclude that OT has beneficial effects on healing and preventive effects on the generation of scar formation in experimental APN of rat.

Procalcitonin (PCT), a prohormone of calcitonin, was initially reported to be a potential marker of bacterial infections in patients with sepsis and infection [48]. However, more recent reports suggest that PCT levels are correlated with the presence of renal scarring after a first UTI episode in febrile children [27]. From this point, we aimed to evaluate PCT levels of serum and we found that PCT levels were significantly increased in the APN group. On the other hand, PCT levels were significantly decreased in the treatment groups which show a clear correlation with histopathologic injury evaluation. Measurement of serum PCT is also used to diagnose bacteremia, since PCT is released in response to bacterial infection. Therefore, our results also displayed that neither antibiotherapy nor OT was effective in preventing bacterial infection in our experimental model.

We also aimed to evaluate serum NP levels of animals. Neopterin is formed during the course of cell-mediated immune response and there is a strong correlation between detection of neopterin and the ability of monocytes/macrophages to scavenge ROS [28]. Thus, NP determination may be considered as both an indirect marker for the amount of immunologically-induced oxidative stress and the effects of therapeutic interventions which are assigned to interfere with the degree of immune activation. According to serum NP levels, APN causes immunologically-induced oxidative stress and OT is found to be effective possibly through affecting cell immunity.
Our results show that neither antibiotic nor OT is sufficient to reduce oxidative stress and renal inflammation. Interestingly, the combination of antioxidant therapy and OT markedly ameliorated renal dysfunction and improved antioxidant status and histopathologic modalities in rats subjected to *E. coli*-induced APN than either antibiotic or OT treatment alone. Based on the fact that both bacterial invasion and oxidative/nitrosative stress may play important roles in the pathogenesis of renal lesions, we believe that additional treatment modalities should be added to classical antibiotic therapy. The findings in the current study show that OT is a very good candidate. Therefore, OT may be considered as an adjuvant therapy to classical antibiotic therapy to prevent renal inflammation in APN.

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

**References**


