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Preliminary study on ozone hemotherapy for canine ischemic renal failure

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Abstract: In dogs, kidney injury is a sudden deterioration in renal function that can be fatal. Ischemia, inflammation, nephrotoxin exposure, and infectious diseases are the most frequent causes. In several fields of regenerative medicine, ozone therapy has recently attracted considerable attention. Clinical studies have shown that ozone therapy is safe and effective, and that it reduces readmission rates for kidney injury in animal models of renal disorders. This study investigated the efficacy of ozone therapy in experimentally induced ischemic renal failure in dogs. Ten dogs were used in this study. All dogs underwent right nephrectomy and 60 minutes of left renal ischemia followed by reperfusion. The animals were divided into two equal groups. The control group received no treatment, and the O3 group received an ozone–oxygen mixture. All groups were evaluated clinically and biochemically until day 40 after surgery, after which they underwent histopathological assessment. Major ozonated autologous hemotherapy substantially reduced the degree of elevation in the treatment group's serum blood urea nitrogen and creatinine levels relative to the control group. Medical ozone therapy considerably reduced the degree of glomerular filtration rate decline and notable alterations; it resulted in a morphological improvement in renal tubular structure compared with that in the control group, and a well-structured improvement in glomeruli, which were reasonably close to those in the healthy control group. According to the findings, ozone therapy may be an effective way to enhance renal function. However, our results indicate that the aforementioned treatment has potential as a straightforward therapeutic strategy for the management of renal failure that may be caused by ischemia or other factors.
Keywords: Acute Renal Failure, Autohemotherapy, Dogs, Medical ozone, Nephrectomy.

1. Introduction

Renal diseases, which are common in dogs and often associated with a poor prognosis in the late stages, can cause abrupt declines in renal function. About 2%–5% of all dogs suffer from renal failure, one of the most dangerous issues in the canine population that can be gradually brought on by renal diseases (Kumar *et al*., 2023).

Ischemic renal failure occurs when there is a brief disruption and subsequent restoration of blood flow to the kidney. It frequently occurs in clinical settings and could include renal artery blockage, heart surgery, and renal transplantation (Shen *et al*., 2024). It causes nitrogenous wastes to be retained in plasma and a sharp decline in renal function. Dogs frequently contract the illness, which has a high morbidity and fatality rate (Cao *et al*., 2020). Renal failure has a highly complex pathophysiology that includes adenosine triphosphate (ATP) depletion, intracellular Ca+2 and reactive oxygen species buildup, proinflammatory cytokine release, and activation of the apoptotic pathway (Yang *et al*., 2023). Acute renal injury in dogs frequently necessitates extended hospital stays, which can be expensive, and they face the risk of acquiring chronic kidney disease (Chen *et al*., 2023).

Ozone has an antioxidant defense system and medicinal qualities that modulate apoptosis. Although many techniques can be used to administer medical ozone, major ozonated autohemotherapy is the most dependable and sophisticated method (Yang *et al*., 2023; Luo *et al*., 2023). Depending on the intended therapeutic goal, ozone therapy can be administered at doses ranging from 1 µg/mL to 100 µg/mL, with safety and efficacy, through various methods (Serra *et al*., 2023). O3 dose methods include topical and infiltrative therapy (for localized effects, such as musculoskeletal and germicidal), autohemotherapy, and rectal insufflation (for systemic effects), and they vary according to the objectives and site of therapy (Hidalgo-Tallón *et al*. 2022). Ozone therapy reduces oxidative stress and inflammation, which are implicated in organ damage in chronic illnesses (Lino *et al*., 2024).

Moreover, ozone increases the activity of enzymes such as hydrogen peroxide, oxidized glutathione reductase, and superoxide dismutase by efficiently eliminating free radicals, thereby enhancing local tissue metabolism, stimulating fibroblast proliferation, facilitating collagen fiber synthesis, and supporting angiogenesis (Wen and Chen, 2020). Ozone therapy facilitates the secretion of growth factors by macrophages and fibroblasts, promoting angiogenesis and granulation tissue formation, thereby accelerating tissue healing. The therapeutic mechanism of ozone can be attributed to the upregulation of growth factors, including vascular endothelial growth factor, transforming growth factor beta, and platelet-derived growth factor (Sun *et al*., 2024). It increases blood flow and oxygen transport in ischemic tissues (Emre *et al*., 2024). Xanthine oxidase is thought to mediate oxidative damage during renal ischemia. When cells are subjected to ischemia, this enzyme breaks down nucleotides.

Nitric oxide (NO), when supported by ozone therapy, can be a defense mechanism against endothelin-1-induced kidney damage, inflammation, and vasoconstriction (Delgadillo-Valero *et al*., 2023). Ozone never exceeds 5% of the gaseous mixture when therapeutic oxygen is used as the carrier; the highest ozone concentrations in this context are estimated at roughly 100 mg/L (Travagli *et al*., 2023). Ozone therapy works by boosting cellular metabolism, decreasing the production of proinflammatory prostaglandins and allogenic compounds, increasing the release of immunosuppressive cytokines, reducing oxidative stress by inducing the

synthesis of antioxidant enzymes, increasing the amount of oxygen that reaches tissues, and stimulating angiogenesis (Sumida *et al*., 2022). Ozonated blood can activate endothelial cells, causing them to release NO. As a well-known vasorelaxant, NO dilates vascular walls and exerts positive effects, especially in hypoxic tissues (Boczkowska *et al*., 2022). According to the study's hypothesis, ozone therapy may restore and enhance renal function and serve as a viable alternative solution to renal failure.

The use of medical ozone in the treatment of various kidney diseases has been the subject of recent clinical research. However, research on the use of medical ozone in the treatment of renal failure is scarce. Therefore, the purpose of this study is to evaluate the medical use of ozone in the regeneration and function of kidneys affected by ischemic renal failure.

2. Materials and Methods

2.1. Ethics Statement

The current study was approved by the Veterinary Medical College's Animal Ethics Committee at the University of Basrah (Approval No. 63/2024). Physical examination, complete blood count (CBC), and serum biochemical analysis were performed on all dogs to check for systemic diseases and confirm normal renal function. Each dog was vaccinated against rabies and given antiparasitic drugs. Food and drink were also readily available.

2.2. Experimental Design

Ten adult male mongrel dogs aged 1–1.5 years were used in this study. The body weights of the animals were extended from 18 kg to 22 kg, with a mean of 20 kg. The right kidneys of all dogs underwent nephrectomy, and the left kidneys were subjected to renal ischemia by clamping the renal artery and vein for 60 min. The animals were divided randomly into two equal groups as follows: Group I: The dogs were treated with an ozone/oxygen gas mixture administered via major ozonated autohemotherapy. Group II: The dogs received no treatment. All dogs were euthanized 40 days after surgery.

2.3. Surgical Procedure

Induction of unilateral renal ischemia reperfusion (I/R):

The procedure was implemented in a sterile environment. All animals were denied food for 10 h and allowed unrestricted access to water until 5 h before the procedure. Each dog received prophylactic antibiotic (pen-strep, 8 mg/kg) intramuscularly 1 h before surgery.

A combination of 2% xylazine hydrochloride at 5 mg/kg body weight (intramuscular injection [I/M]) and 10% ketamine hydrochloride at 10 mg/kg body weight (I/M) was used to anesthetize the dogs. To be ready for aseptic surgery, the dogs were placed in the dorsal position, and the surgical site, which runs from the xiphoid cartilage to the pubic bone, was cleaned and trimmed. An abdominal incision was made in the midline.

The bowels were retracted upon entry into the peritoneal cavity, and an atraumatic vascular clamp was used to cross-clamp the renal artery and vein of each dog's left kidney to cause ischemic injury. After 60 min, the clamp was released to allow for reperfusion.

Successful ischemia or reperfusion was assessed by tracking the change in tissue color from red to navy blue or from navy blue to red, respectively. Meanwhile, the right kidneys underwent nephrectomy.

During the surgical procedure, all dogs were administered intravenously with standard saline solution (10 mL/kg/h). The surgical wound was closed using polydioxanone (PDS II, size 2-0), followed by a continuous suture pattern. Then, the skin was closed with an interrupted suture pattern using a nylon suture (size 2-0). Throughout the planned study period, each animal was kept in a separate cage. In addition to daily medical care after surgery, the dogs were given injectable penicillin streptomycin for four days at doses of 10,000 IU and 20 mg/kg body weight. The dogs were then given unrestricted mobility. Fourteen days after the procedure, the wound sutures were removed. Every dog underwent clinical, biochemical, and histological evaluation (Cao *et al.,* 2020).

2.4. Biochemical Analysis

Creatinine (SCr) and blood urea nitrogen (BUN) levels were used to evaluate renal function. Five milliliters of blood were drawn from each dog's cephalic vein before surgery, two days after surgery, and every 10 days after treatment to ascertain the serum chemical profile. The serum BUN and SCr reference ranges were 8–30 and 0.5–1.5 mg/dl, respectively. SCr and serum BUN levels exceeding the standard limits were deemed abnormal (Lee *et al*., 2017).

2.5. Clinical Evaluation

All dogs were observed to evaluate the clinical signs on days 10, 20, 30, and 40 postoperatively.

2.6. Major Ozonated Autohemotherapy

Two days after surgery, each dog's cephalic vein yielded 25 mL of blood, which was then placed in a sterile transfusion blood bag with 13 mL of sodium citrate (3.8%, 380 mg/mL). The animal's weight was used to determine the blood volume (25 mL). The blood bag was filled with the oxygen–ozone mixture at a dose of 37.3 ug/mL. A generator with a production capacity of 0.00023 g/min, fueled by a 99.5% pure oxygen ampoule at a pressure of roughly 250 kgf/cm^2 and a flow rate of 3 L/min, produced the oxygen–ozone combination. The blood was reintroduced into the animal via the cephalic vein after ozonation and gentle shaking for 5 min, until the foam disappeared and the blood turned bright red (Sumida *et al*., 2023; Sancak *et al*., 2017).

2.7. Kidney Harvest

After 40 days of treatment, the dogs' left kidneys were promptly removed, and the dogs were euthanized. The renal tissue samples were preserved in 10% neutral-buffered formalin for histological analysis (Aum *et al*., 2023).

2.8. Histopathology Evaluation

On day 40 after surgery, renal tissue was taken from the left kidney's caudal pole, preserved in 10% buffered formalin, embedded in paraffin, and cut into sections that were 3 µm thick. The sections were stained with hematoxylin and eosin to assess histological alterations (Liu *et al*., 2023).

3. Statistical Analysis

The results were expressed as mean values–standard errors. Data were statistically analyzed using an independent-samples t-test and a one-way analysis of variance (ANOVA) with multiple-comparison tests in a statistical software program (SPSS for Windows, version 22, USA). Differences were considered significant at $P \leq 0.05$.

4. Results

4.1. Clinical Evaluation

The most common clinical signs that were observed postoperatively in all the dogs were lethargy, anorexia, vomiting, diarrhea, polyuria, and polydipsia.

Clinical symptoms observed after surgery occurred in all animals during the first 10 days after treatment, but they gradually began to disappear in the O3 group. In the control group, the signs continued until the 20th day, then appeared intermittently until they gradually disappeared after the 30th day.

4.2. Biochemical Evaluation

Major ozonated autohemotherapy notably reduced serum BUN and SCr elevations compared with the control group at 10, 20, 30, and 40 days after treatment in ischemic renal failure. The levels of urea in the O3 group on days 10, 20, 30, and 40 were 379,778, 200,262, 872, and 46,146 mg/dL, respectively, whereas those in the control group were 364,298, 310,302, 264,81, and 214,546 mg/dL, respectively. The levels of SCr in the O3 group on days 10, 20, 30, and 40 were 4.842, 2.91, 2.298, and 1.654 mg/dL, respectively, whereas those in the control group were 4.422, 3.91, 3.412, and 2,846 mg/dL, respectively, as shown in Figures 1 and 2 (Aum *et al*., 2022).

4.3. Histopathology Evaluation

The histopathological sections of the kidneys from the positive control group showed severe glomerular atrophy and cystic dilation of renal tubules (Figure 3). The O3 group showed marked glomerular structural and morphological improvements in renal tubules compared with the control group (Figure 4). The O3 group also showed remarkable changes, representing a highly structural improvement in glomeruli, approximating those of the healthy control, and a morphological improvement in renal tubules compared with the control group (Figure 5).

5. Discussion

Medical ozone is utilized in human and veterinary medicine despite its status as a pollutant. Ozone therapy, which involves the medicinal use of an ozone/oxygen gas mixture, is based on the idea that ozone quickly dissociates into water and releases a reactive form of oxygen that can oxidize cells. This dissociation increases the amount of ATP and oxygen available for cellular activity. Systemic and local routes are the two primary methods for delivering ozone. Ozone autohemotherapy, which involves *ex vivo* injection of a specific concentration of oxygen–ozone into a preset volume of blood, is used for systemic administration. Afterward, the patient receives this oxygenated-ozonated blood (Sciorsci *et al*., 2020).

Ozone therapy improves the body's antioxidant responses by causing regulated oxidative stress. However, little is known about how this treatment affects canine renal failure. Our goal was to evaluate the clinical, biochemical, and clinical characteristics of dogs affected with renal failure receiving autohemotherapy with oxygen and ozone (Oliveira *et al*., 2024). Ozone therapy's clinical trials are still in their infancy, despite preclinical research suggesting it may be effective for treating renal ailments. In accordance with Boczkowska-Radziwon *et al*. (2022), substantial ozonated autohemotherapy was used in this investigation because ozonated blood can activate endothelial cells via lipid peroxidation products and drive them to generate NO, which dilates the vessel walls and has positive effects, especially in hypoxic tissues.

Clinical symptoms, such as diarrhea, that resulted from direct gastrointestinal injury brought on by the presence of uremic toxins were observed in all groups in this study after the induction of ischemic renal failure. These symptoms are consistent with those reported by Rimer *et al.* (2022). Systemic hypertension and decreased medullary concentration capacity may be the cause of polyuria and polydipsia. Lack of appetite and reduced feed intake may cause anorexia and weakness, and intestinal malabsorption and uremic catabolism may induce weight loss and weakness. Moreover, uremic toxins may cause vomiting by stimulating the medullary emetic chemoreceptor trigger zone, leading to gastroenteritis, as explained by Pathak *et al.* (2023).

The histopathological sections of the kidneys from the positive control group showed severe glomerular atrophy and cystic dilation of renal tubules, resulting from ischemic changes in the renal parenchyma. These results are consistent with those of Dunaevich *et al*. (2020), who reported that ischemia is a significant cause of acute renal failure. In the current study, renal failure

was induced via the occlusion of the renal blood supply for 60 min with atraumatic clamps. These results are also consistent with those of Fan *et al*. (2023), who explained that damage to the renal parenchyma, glomeruli, renal tubules, renal interstitium, and renal microvessels is the consequence of ischemic renal failure. Compared with the control group, the ozone group showed glomerular morphological improvement and mild renal tubular dilation, reflecting morphological recovery following ischemic changes in the renal parenchyma.

The ozone group showed marked structural improvement in glomeruli and morphological improvement in renal tubules compared with the control group, reflecting recovery from ischemic changes in the renal parenchyma. This result can be explained by the finding of Gan *et al*. (2023), who reported that ozone may freely diffuse into RBCs and has a strong antioxidative stress effect. Activating phosphofructokinase can increase ATP and 2,3-DPG levels, improve blood oxygen delivery to hypoxic tissues, and exert an anti-inflammatory effect by reducing the synthesis of inflammatory factors, such as TNF-α, IL-1β, and IL-6. Moreover, ozone helps treat ischemia-reperfusion injury in organs. The ozone group in the current study showed substantial changes, representing a significant structural improvement of the glomeruli, approaching those of the healthy kidney, and a morphological improvement of renal tubules compared with the control group, due to complete morphological recovery following ischemic changes in the renal parenchyma. These results confirm the effects of ozone therapy on kidney tissue during treatment and align with those of Sun *et al.* (2024), who reported that ozone plays a vital role in regulating cellular proliferation and accelerating tissue repair. It can also help macrophages and fibroblasts secrete growth factors that promote angiogenesis.

Our results also align with those of Sumida et al. (2022), who reported that ozone therapy stimulates angiogenesis, increases the release of immunosuppressive cytokines, decreases the production of proinflammatory prostaglandins, increases oxygen delivery to tissues, and activates cellular metabolism. Enhancing erythrocyte pliability and potentially reducing blood viscosity and erythrocyte aggregation improve tissue oxygenation and increase renal blood flow during reperfusion in an organ that has previously experienced inadequate blood flow. The ozone group in the current study was highly superior to the acute renal failure control group.

Ten, twenty, thirty, and forty days after treatment for ischemic renal failure, major ozonated autohemotherapy substantially decreased the levels of blood BUN and SCr compared with those in the control group. As previously mentioned, the benefits of ozone therapy led to this improvement in renal function, as evidenced by a decrease in BUN and SCr levels.

6. Conclusions

The results of our investigation indicate that ozonated hemotherapy has potential as a straightforward therapeutic strategy for the management of renal failure that may be caused by ischemia or other factors.

Our study has several limitations. First, the dogs were followed up for only 40 days. Long-term studies are needed to evaluate the effectiveness of ozone therapy in ischemic renal failure and to determine whether it can prevent progression to chronic kidney disease. Second, the dogs were administered a single fixed dose concurrently with ischemia. Repeated administration of such therapy may be synergistic.

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