

# Harvesting Model in Rats: Investigation of the Protective Effect of Ozone in Cold Ischemic Renal Injury and the Evaluation of the Damage

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## What's known on the subject? and What does the study add?

Cold ischemia causes oxidative and structural damage in harvested kidneys, which negatively affects graft quality. Preservation solutions, including the University of Wisconsin Solution, are routinely used to reduce ischemic injury; however, oxidative stress remains a major challenge during organ preservation. This experimental rat harvesting model demonstrates that intraperitoneal ozone administration before organ removal may reduce oxidative damage and histopathological injury during cold ischemia, suggesting a novel protective strategy to improve kidney preservation.

## Abstract

**Objective:** We aim to investigate cold ischemic injury in the kidney harvested from rats and the protective effect of ozone (O<sub>3</sub>) on cold ischemia damage.

**Materials and Methods:** Thirty Wistar albino male rats were randomly assigned to three groups. Group 1 received isotonic sodium chloride (n=10; I), Group 2 received the University of Wisconsin Solution (n=10; UW), from the aorta during the procedure. Group 3 (n=10; IP + UW) was given O<sub>3</sub>-oxygen mixture at a dose of 0.7 mg/kg (50 µg/mL) intraperitoneally 30 minutes before the procedure, in addition to the UW Solution. The organs were excised and stored in the given solutions for 12 hours. Thereafter, the kidneys were removed for examination.

**Results:** The biochemical evaluation revealed significantly lower levels of advanced oxidation protein products and protein carbonyl groups in the IP + UW group than in the I group. Statistically, these general protein oxidation indicators were also lower in the IP + UW group compared to the UW group. Superoxide dismutase levels were significantly higher in the IP + UW group than in the UW group. Histopathologically, the number of degenerated or necrotic tubules (D/NT), the number of tubules exhibiting brush border loss (BBT), and the number of tubular cast structures were significantly higher in group I than the UW and IP + UW groups. Comparison of the UW and IP + UW groups showed that the D/NT and the BBT counts were significantly higher in the UW group.

**Conclusion:** Intraperitoneal O<sub>3</sub> therapy, together with UW Solution, effectively attenuated cold ischemic injury in rat kidneys.

**Keywords:** Organ harvesting, intraperitoneal injections, ozone, rats, cold ischemia

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## Introduction

Chronic kidney disease (CKD) is a multifactorial pathophysiological condition characterized by a gradual and irreversible decline in nephron number and function, frequently progressing to end-stage kidney disease (ESKD). Following CKD, many patients eventually develop ESKD, a stage in which intrinsic renal function is permanently lost. This life-threatening condition necessitates renal replacement therapies, including long-term dialysis or kidney transplantation, to prevent uremic complications (1). Kidney transplantation remains the most effective therapeutic option for ESKD patients requiring dialysis (2).

The preservation of the excised organ in protective solutions until transplantation (cold ischemia) and the potential injury caused by inadequate nutrient supply during this period represent critical factors that can negatively affect transplantation outcomes (3). Ozone ( $O_3$ ) therapy consists of delivering a controlled mixture of  $O_3$  and oxygen ( $O_2$ ) into the body (4). Research indicates that  $O_3$  therapy may enhance the activity of the antioxidant system, thereby mitigating damage induced by oxidative stress (5).  $O_3$  therapy has been reported to provide beneficial effects on renal ischemia-reperfusion (I/R) injury (6).

In this study, renal cold ischemic injury following organ procurement in rats and the potential protective role of  $O_3$  against ischemia-related damage were evaluated.

## Materials and Methods

30 male Wistar albino rats (350–400 g) were housed in specialized cages under standard feeding conditions for the duration of the study. All rats underwent clinical evaluation, including behavioral, respiratory, and cardiovascular assessments, and

were subsequently included in the study because no adverse events were observed. During the study period, every rat was provided with a standard chow diet and free access to water. Each rat was maintained in an environment set at  $21 \pm 2^\circ\text{C}$ , under a 12:12-hour light-dark cycle. The rats were individually housed in cages within a clean-air environment. Food was provided ad libitum, except during an 8 hours fasting period prior to anesthesia.

The experimental rats were randomly allocated to three groups of ten animals each. All subjects received anesthesia. A midline incision was made to access the rats' abdominal cavities (Figure 1). Cannulation of the aortas was performed caudal to the renal arteries in all rats, and the aortas were ligated cranial to the renal arteries. During the procedure, Group 1 was administered isotonic sodium chloride (NaCl) ( $n=10$ ; I) via the aorta. Group 2 received the University of Wisconsin Solution ( $n=10$ , UW), which is routinely employed for organ retrieval from the aorta (Figure 2). In addition to the UW Solution administered from the aorta, Group 3 ( $n=10$ ; IP + UW) received an intraperitoneal  $O_3$ - $O_2$  mixture at a dose of 0.7 mg/kg (50  $\mu\text{g/mL}$ ) 30 minutes before the procedure to allow sufficient time for  $O_3$ -induced oxidative preconditioning and activation of endogenous antioxidant defense mechanisms prior to ischemia, as previously described in experimental studies (6,7). The organs from all three groups were harvested according to standard procedure, and the rats were euthanized upon its completion. After 12 hours of storage in the designated preservation solutions at  $4^\circ\text{C}$ , the harvested organs were collected for biochemical and histopathological analyses. The experimental procedure was then concluded.

At the end of the experiment, the harvested kidney tissues were divided into two portions. The first portion of each kidney was stored in either isotonic NaCl or UW solution for 12 hours. It was

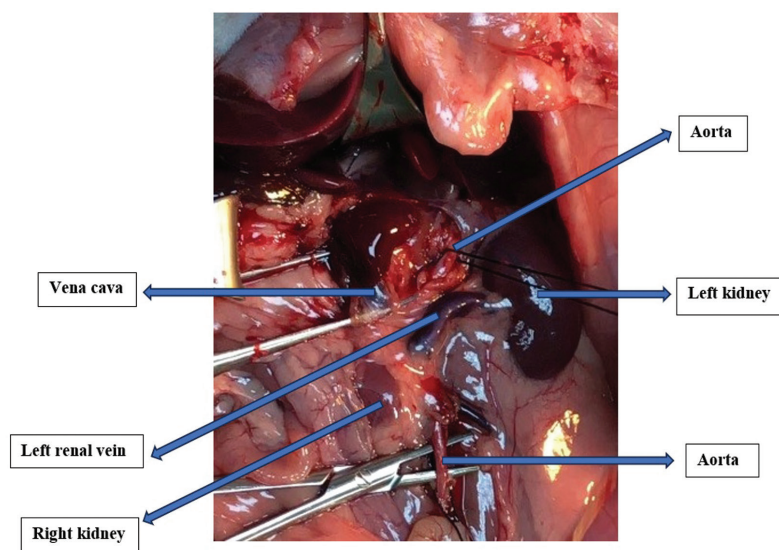


Figure 1. Exposure of the aorta, vena cava, and renal veins

then aseptically dried and preserved at  $-86^{\circ}\text{C}$  until biochemical analyses. These analyses included advanced glycation end products, advanced oxidation protein products (AOPP), and protein carbonyl groups (PCO). Malondialdehyde, kynurenine (KYN), lipid hydroperoxides, N-formyl KYN (N-FKYN), superoxide dismutase (SOD), and total thiol fractions were also quantified. The second portion of each kidney was stored in either isotonic NaCl or UW Solution for 12 hours, after which it was transferred to formalin for histopathological evaluation.

### Biochemical Method

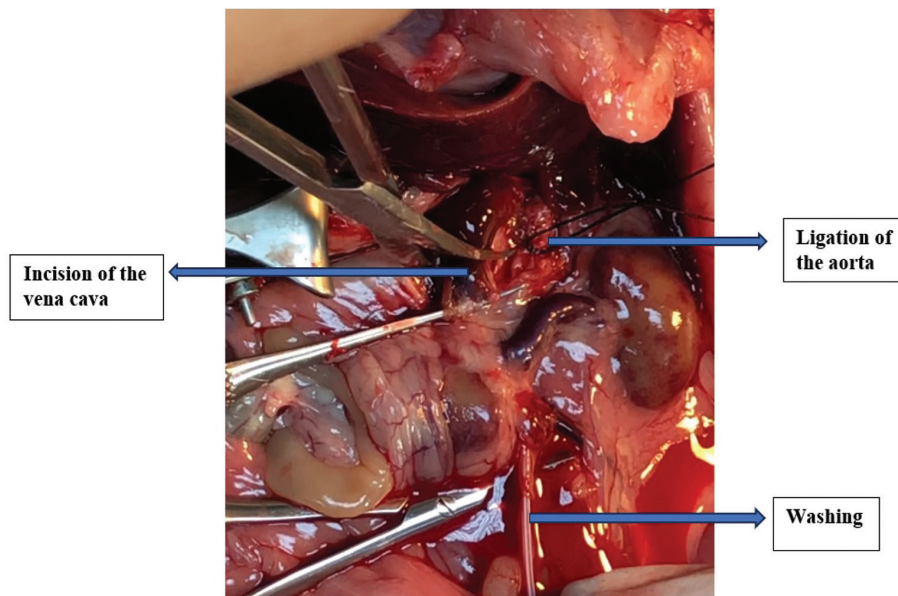
Biochemical analyses were conducted by an experienced biochemist unaware of the group assignments. The tissues were maintained at  $-86^{\circ}\text{C}$ . Before analysis, they were removed from the freezer. Kidney tissue specimens were rinsed in chilled 0.9% NaCl and placed on an ice-cold plate. After dissection, their masses were determined. The specimens were promptly frozen in liquid nitrogen and stored until homogenization. Next, they were manually homogenized in a buffer ( $100\text{ mM KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ ) to prepare 10% homogenates. Homogenization was performed on ice using a tissue grinder equipped with a Teflon pestle. The homogenates were sonicated twice on ice at 30 seconds intervals. In this step, a MSE sonicator with a power output of 38 W was used. The homogenates were centrifuged at  $15,000\text{ g}$  for 15 minutes. The obtained supernatant was collected for further analysis. Throughout the aliquoting process, the supernatant samples were kept at  $4^{\circ}\text{C}$  under low-light conditions. The supernatant samples were aliquoted. Each aliquot was designated for a specific assay. The samples were stored at  $-80^{\circ}\text{C}$  for no longer than two weeks prior to biochemical analyses (8-15).

### Histopathological Method

An experienced pathologist, unaware of the treatment allocation, performed the morphological evaluation. Kidney samples collected from the subjects were fixed in 10% buffered formalin and individually placed into cassettes for morphological analysis. The kidney tissues were processed using an automated tissue processor (Leica TP 1020) and embedded in paraffin. Kidney tissues were sectioned into  $4\text{-}\mu\text{m}$ -thick slices. The sections were stained using hematoxylin and eosin (H&E). A light microscope (Leica DM2500) was used to observe the morphological features. Microscopic assessment was performed following the method described by Pegues et al. (16), without employing an imaging software. The numbers of degenerated or necrotic tubules (D/NT), of tubules exhibiting brush border loss (BBT) without evident cellular injury, and of tubules containing cast formations (TCT) were quantified. D/NT and BBT evaluations were obtained by averaging the counts of tubules exhibiting each injury across 10 random high-magnification fields at the cortex and cortex-medulla junction. TCT assessment was obtained by averaging the number of tubules containing tubular cast structures across 10 randomly selected high-magnification fields at the corticomedullary junction and in the medullary area. Areas with edge and section artifacts were not included in the evaluation.

### Ethics Statement

All experimental procedures were performed at the Experimental Research Application and Research Center of Çanakkale Onsekiz Mart University (ÇOMÜ) following approval from the Animal Experiments Local Ethics Committee of ÇOMÜ (approval number: 2020/07-02, date: 24.08.2020). It was conducted in accordance



**Figure 2.** Ligation of the aorta, initiation of washing from the cannulated aortic section, and incision of the vena cava

with the United Kingdom Animals (Scientific Procedures) Act 1986 and its associated guidelines, and with the European Union (EU) Directive 2010/63/EU for animal experiments.

### Statistical Analysis

Data analysis was conducted using IBM SPSS version 25 (SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was regarded as statistically significant for all analyses. All values are reported as means accompanied by their standard deviations (SD). Following verification of normality using the Shapiro-Wilk test, normally distributed data were analyzed with a parametric one-way analysis of variance. For parameters showing significant differences, group comparisons were performed using a post-hoc test. Non-normally distributed parameters were analyzed using the Kruskal-Wallis test. Pairwise comparisons of significant parameters were performed using a post-hoc test.

## Results

### Biochemistry

Levels of AOPP and PCO, recognized as indicators of general protein oxidation, were significantly reduced in the IP + UW

group versus the I group ( $p < 0.05$ ). Though the UW group demonstrated a downward trend, the change did not reach statistical significance. A more marked reduction in general protein oxidation markers occurred in the IP + UW group relative to the UW group ( $p < 0.05$ ).

SOD activity exhibited a modest, non-significant rise in the IP + UW group compared with the I group ( $p > 0.05$ ); however, SOD activity levels were markedly greater than in the UW group ( $p < 0.05$ ), suggesting a possible antioxidant response. In the UW group, SOD levels declined compared with levels in the I group; however, this difference failed to achieve statistical significance ( $p > 0.05$ ) (Table 1).

### Histopathology

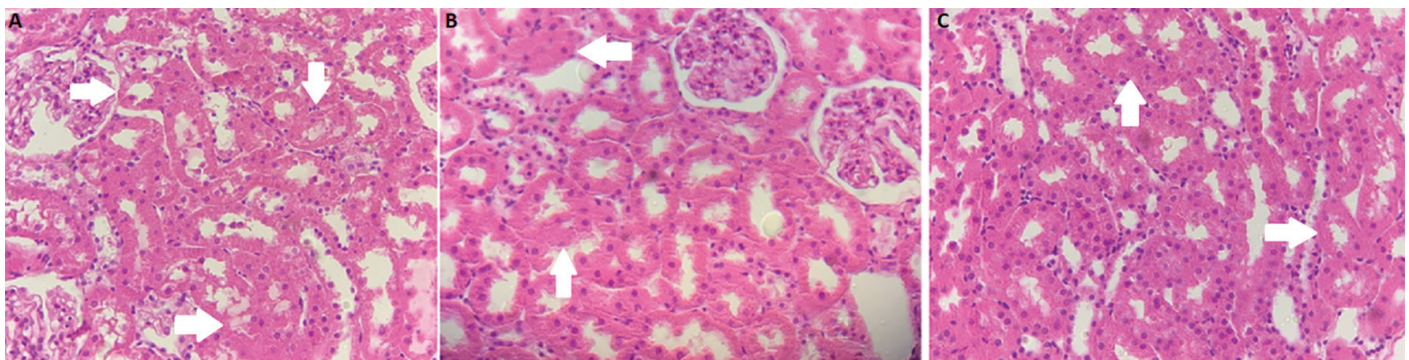
Light microscopy revealed clear, diffuse signs of acute tubular injury in the I group, whereas the UW and IP + UW groups displayed milder tubular damage than the I group. Images of the H&E-stained sections are presented in Figures 3 and 4. Table 2 presents the mean, minimum, and maximum values, along with SDs, of the parameters assessed across the groups.

**Table 1. Comparison of oxidative stress parameters of I, UW and IP + UW groups (n=10 per group)**

|         |      | KYN             | N-FKYN          | AOPP                   | PCO               | LHPs                           | MDA               | AGEs            | T-SH              | Cu, Zn-SOD activity |
|---------|------|-----------------|-----------------|------------------------|-------------------|--------------------------------|-------------------|-----------------|-------------------|---------------------|
|         |      | (FU/mg protein) | (FU/mg protein) | ( $\mu\text{mol/L}$ )* | (nmol/mg protein) | ( $\mu\text{mol/mg protein}$ ) | (nmol/mg protein) | (FU/mg protein) | (nmol/mg protein) | (U/mg protein)      |
| I       | Mean | 658.65          | 527.62          | 55.87                  | 4.61              | 0.67                           | 3.50              | 490.13          | 9.57              | 2.78                |
|         | SD   | 194.13          | 118.51          | 14.37                  | 1.05              | 0.09                           | 0.86              | 89.28           | 1.35              | 1.91                |
| UW      | Mean | 674.72          | 535.82          | 50.73                  | 4.26              | 0.66                           | 5.50              | 506.55          | 9.48              | 1.71                |
|         | SD   | 102.32          | 44.94           | 8.66                   | 0.42              | 0.11                           | 0.96              | 65.94           | 0.65              | 0.64                |
| IP + UW | Mean | 806.01          | 604.50          | 32.13                  | 2.73              | 0.62                           | 5.24              | 584.47          | 10.36             | 3.08                |
|         | SD   | 71.23           | 51.72           | 6.78                   | 1.10              | 0.10                           | 0.86              | 18.90           | 1.31              | 0.65                |

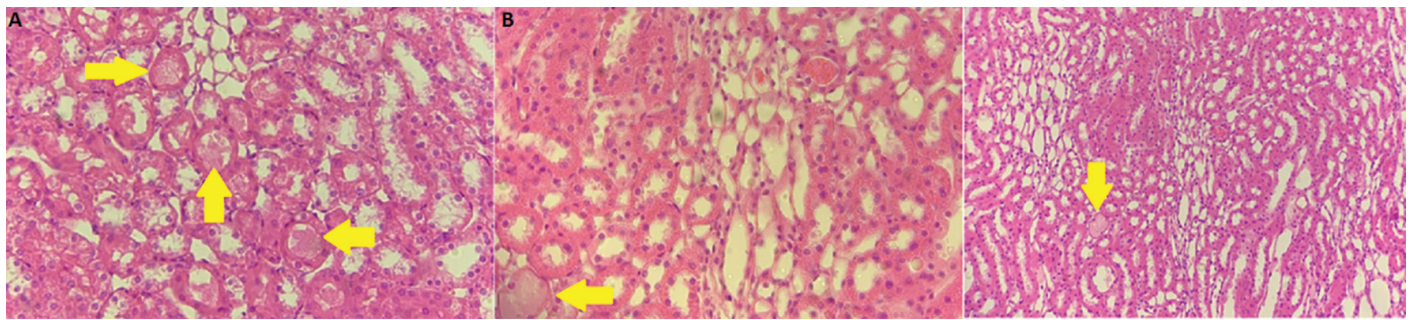
\*Values are expressed as Chloramine-T equivalents

SD: Standard deviation, KYN: Kynurenine, N-FKYN: N-formyl KYN, AOPP: Advanced oxidation protein products, PCO: Protein carbonyl groups, LHP: Lipid hydroperoxide, MDA: Malondialdehyde, AGE: Advanced glycation end product, T-SH: Total thiol fractions, Cu, Zn-SOD: Superoxide dismutase, I: Isotonic sodium chloride group, UW: University of Wisconsin Solution group, IP + UW: Intraperitoneal  $\text{O}_3$  + University of Wisconsin Solution group



**Figure 3.** Degenerated/necrotic tubules marked with white arrows in kidney tissues belonging to I, UW and IP+UW groups, from left to right, at low magnification (H&E, 400x)

H&E: Hematoxylin and eosin, I: Isotonic sodium chloride group, UW: University of Wisconsin Solution group, IP + UW: Intraperitoneal  $\text{O}_3$  + University of Wisconsin Solution group



**Figure 4.** Cast-containing tubules marked with yellow arrows in kidney tissues belonging to I, UW (H&E, 400x) and IP + UW groups, from left to right, at low magnification (H&E, 200x)

H&E: Hematoxylin and eosin, I: Isotonic sodium chloride group, UW: University of Wisconsin Solution group, IP + UW: Intraperitoneal O<sub>3</sub> + University of Wisconsin Solution group

**Table 2. Minimum, maximum and mean values and standard deviations of D/NT count, BBT count and TCT count in I, UW and IP + UW groups**

| Values                    | I        | UW       | IP + UW  |
|---------------------------|----------|----------|----------|
| <b>D/NT count</b>         |          |          |          |
| Minimum - maximum         | 55-82    | 22-39    | 18-26    |
| Mean - standard deviation | 66.7+9.2 | 32.7+5.9 | 22.1+2.6 |
| <b>BBT count</b>          |          |          |          |
| Minimum - maximum         | 7-19     | 42-53    | 49-63    |
| Mean - standard deviation | 16.6+3.8 | 47.2+3.8 | 57.1+4.3 |
| <b>TCT count</b>          |          |          |          |
| Minimum - maximum         | 2-8      | 1-2      | 1-2      |
| Mean - standard deviation | 4.4+1.8  | 1.3+0.5  | 1.2+0.4  |

D/NT: Degenerated or necrotic tubules, BBT: Tubules exhibiting brush border loss, TCT: Tubules containing cast formations, I: Isotonic sodium chloride group, UW: University of Wisconsin Solution group, IP + UW: Intraperitoneal O<sub>3</sub> + University of Wisconsin Solution group

### Statistical Analysis

Statistical analysis showed that the numbers of D/NT, BBT, and TCT in the I group exceeded those noted in the UW group ( $p<0.001$  for all comparisons). Similarly, the numbers of D/NT, BBT, and TCT were significantly greater in the I group than in the IP + UW group ( $p<0.001$  for all comparisons). The number of tubules observed within a microscopic field falls within a defined range. The tubules observed in this field represent the total of those categorized as D/NT and as BBT. As shown in Table 2, the BBT count was lower in group I than in the other groups. This was attributed to the higher number of D/NT in the I group than in BBT. To summarize the data presented in Table 2, the lower BBT count in the I group was primarily due to a high degree of damage, i.e., an increased number of D/NT.

A comparison of the UW and IP + UW groups indicated that D/NT ( $p=0.001$ ) and BBT ( $p=0.001$ ) counts were significantly higher in the UW group, whereas TCT counts showed no significant difference between groups.

### Discussion

Renal transplantation is influenced by multiple perioperative variables that may compromise graft function. Among these, cold ischemia and subsequent reperfusion represent major sources of allograft injury. Reactive O<sub>2</sub> species (ROS) and proinflammatory mediators are considered key contributors during the reperfusion phase (17). Accordingly, strategies aimed at limiting ROS generation and attenuating inflammatory mediator release may contribute to improved graft performance and prolonged allograft viability. Our findings demonstrate that O<sub>3</sub> preconditioning significantly attenuates oxidative stress markers and histopathological injury in rat kidneys, suggesting a protective effect.

Previous studies have demonstrated the protective effects of O<sub>3</sub> against I/R injury across different tissues and organs. Chen et al. (6) evaluated the protective effects of O<sub>3</sub> on renal I/R injury in their post-reperfusion observation studies. Recently, a study in a spinal cord I/R injury model specifically investigating different administration routes (intrathecal, intraperitoneal, and rectal) reported that O<sub>3</sub> could control the adverse effects of I/R injury on renal tissue by regulating cellular oxidative stress mechanisms (7).

AOPP levels, mainly derived from albumin, reflect the degree of protein oxidation. Previous studies have shown that ischemia induces structural changes in serum albumin, leading to the identification of a novel serum marker. Tusat et al. (18) investigated the protective effects of intraperitoneally administered O<sub>3</sub> in preventing testicular I/R injury and found that serum ischemia-modified albumin levels were lower in the O<sub>3</sub> group. Jamel et al. (19) showed that plasma PCO measurement might be useful as an oxidative stress biomarker in intestinal I/R injury. Our study revealed that AOPP and PCO levels, indicators of protein oxidation, were significantly reduced in the IP+UW group compared with those in the I group. A downward trend was observed in the UW group; nonetheless, the change was

not significant. The reduction in general protein oxidation parameters was notably larger in the IP + UW group than in the UW group; this difference reached statistical significance. This shows the protective effect of O<sub>3</sub> in terms of general protein oxidation markers. Our study uniquely demonstrates these protective effects in a cold ischemic renal harvesting model.

SOD activity is upregulated as part of the endogenous antioxidant response to oxidative stress induced by I/R injury (20). In a rat model of ovarian torsion-associated I/R injury, Sayar et al. (21) observed a significant increase in SOD activity following ischemic insult, whereas intraperitoneal O<sub>3</sub> administration mitigated this response, indicating a potential regulatory influence on oxidative stress-mediated antioxidant activation. In two separate experimental models evaluating the protective roles of O<sub>3</sub> and hyperbaric O<sub>2</sub> in skeletal muscle and bone I/R injury, Koca et al. (22,23) reported reduced SOD activity following I/R induction, whereas intraperitoneal O<sub>3</sub> administration significantly enhanced SOD levels. The literature further suggests that SOD activity does not uniformly rise after therapeutic intervention; in some experimental settings, enzyme levels have been reported to remain low despite treatment, highlighting the complex regulation of oxidative stress responses (24). In our study, SOD levels were significantly higher in the IP + UW group than those in the UW group. These findings suggest that O<sub>3</sub> may augment the intrinsic antioxidant defense capacity. This study expands knowledge by suggesting that intraperitoneal O<sub>3</sub> may confer renal protection during cold ischemia by enhancing SOD-mediated antioxidant defenses.

The experimental study of Qiu et al. (25) showed that transrectal administration of O<sub>3</sub> could reduce the rate of oxidative stress damage-induced apoptosis in renal tubular epithelial cells of rats during the renal transplantation process. In the experimental study of Wang et al. (3), the number of tubules with BBT, D/NT, and enlargement was lower in the O<sub>3</sub>-administered rat group compared to other groups. The recent spinal cord I/R study similarly provided histopathological evidence, showing that tubular dilatation and lymphocyte infiltration were statistically reduced in the O<sub>3</sub>-administered rat groups relative to the I/R group. However, that study observed that glomerular vacuolization, vascular vacuolization and hypertrophy, and Bowman capsule dilation were significantly higher in the intrathecal and intraperitoneal groups in relation to the I/R group, indicating that the route of administration may affect specific histological structures differently (7). The histopathological examinations in our study revealed marked increases in D/NT, BBT, and TCT in the I group compared with the UW group. Mirroring prior experimental results, the I group exhibited significantly elevated D/NT, BBT, and TCT counts compared to the IP + UW group in our study. A comparison between the UW and IP + UW groups revealed significantly

higher D/NT and BBT counts in the UW group, whereas TCT counts did not differ significantly. Unlike previous studies investigating rectal O<sub>3</sub> administration in kidney transplant models or other O<sub>3</sub> administration routes in I/R models, our study provides evidence for the histopathological protective effects of intraperitoneal O<sub>3</sub> in a kidney harvesting model under cold ischemia.

### Study Limitations

This study has several limitations. It was conducted in a rat model, and the results may not fully translate to human renal harvesting and transplantation scenarios. O<sub>3</sub> was administered intraperitoneally at a single dose, and alternative dosages or administration routes were not explored, which could influence its protective effects. Proinflammatory mediators, including interleukin-6 (IL-6), tumor necrosis factor-alpha, IL-18, and interferon, were not evaluated, limiting the understanding of the inflammatory mechanisms involved. Future studies are required to address these limitations and clarify the mechanistic effects of O<sub>3</sub> in cold ischemic kidney injury.

### Conclusion

Intraperitoneal administration of O<sub>3</sub> prior to the renal harvesting procedure significantly reduced general protein oxidation and increased SOD levels, indicating enhanced endogenous antioxidant defense. Histopathological examination suggested that intraperitoneal O<sub>3</sub> administration before the procedure appeared to improve most parameters compared to other groups, except the TCT number compared to the UW group. These results support a potential protective effect of intraperitoneal O<sub>3</sub> in a renal harvesting model under cold ischemia in rats. To enable future clinical translation, additional experimental work is required to elucidate the protective mechanisms underlying intraperitoneal O<sub>3</sub> administration and to determine the most effective dosing strategy.

### Ethics

**Ethics Committee Approval:** All experimental procedures were performed at the Experimental Research Application and Research Center of Çanakkale Onsekiz Mart University (ÇOMÜ) following approval from the Animal Experiments Local Ethics Committee of ÇOMÜ (approval number: 2020/07-02, date: 24.08.2020).

**Informed Consent:** Not applicable.

### Footnotes

The manuscript was originally written in Turkish, and then professionally translated and edited into English by protranslate.net Translation Services at the authors' own expense.

## Authorship Contributions

Surgical and Medical Practices: H.U.Ö., H.A.K., E.O.G., C.A., Concept: H.U.Ö., H.A.K., C.A., Design: H.U.Ö., H.A.K., C.A., Data Collection or Processing: H.U.Ö., H.A.K., Y.A.R., E.O.G., K.Y., P.A., Analysis or Interpretation: H.U.Ö., Y.A.R., K.Y., P.A., Literature Search: H.U.Ö., Y.A.R., E.O.G., K.Y., P.A., Writing: H.U.Ö., H.A.K.

**Conflict of Interest:** Cabir Alan Prof. MD is Section Editor in Journal of Urological Surgery. He had no involvement in the peer-review of this article and had no access to information regarding its peer-review. The other authors declared no conflict of interest.

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