Effect of fullerenol C60 on lung and renal tissue in lower extremity ischemia-reperfusion injury in sevoflurane-treated rats

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Received June 21, 2023; Accepted September 1, 2023

DOI: 10.3892/mmr.2024.13178

Abstract. The aim of the present study was to examine the effect of fullerenol C60 on lung and kidney tissue in sevoflurane-treated rats with lower extremity ischemia-reperfusion (IR) injury. A total of 30 Wistar albino rats weighing 225-275 g were used and were equally divided into five groups (n=6/group): i) Sham; ii) IR; iii) IR-fullerenol C60 (IR-FUL); iv) IR-sevoflurane; and v) IR-fullerenol C60-sevoflurane (IR-FUL-SEVO). Fullerenol C60 was administered intraperitoneally prior to lower extremity IR induction and sevoflurane was administered during the IR injury. Subsequently, lung and kidney histopathological examinations, and serum biochemical analyses were performed. Lung tissue showed markedly increased congestion and neutrophil infiltration in the IR group compared with in the sham group, and notable decreases in congestion and neutrophil infiltration were observed in the treatment groups compared with in the IR group. In the histopathological evaluation of the kidney samples, vacuolization, loss of brush border in tubular epithelial cells, tubular epithelial loss and varying degrees of tubular damage were observed in all groups that underwent IR. There was a significant increase in the mean renal tubule injury score in all IR groups compared with that in the sham group. In addition, the mean kidney injury score was significantly lower in the IR-FUL and IR-FUL-SEVO groups than that in the IR group. It was observed that the expression levels of tumor necrosis factor-α, interleukin 1β and intercellular adhesion molecule 1 in the lung and kidney tissues were increased following IR, and were decreased in the groups treated with fullerenol C60 and sevoflurane. Notably, it was determined that the reduction in cytokine expression was greatest in the IR-FUL group. When the oxidant status parameters in the lungs and kidneys were examined, thiobarbituric acid reactive substances levels, and catalase and glutathione S-transferase enzyme activities were significantly different in the groups receiving sevoflurane or fullerenol C60 treatment compared with those in the IR group. The present study demonstrated the protective effects of fullerenol C60 on the lung and kidney tissues of rats under sevoflurane anesthesia after establishment of lower extremity IR. The results of the present study showed that fullerenol C60 can reduce oxidative and histopathological damage in the lungs and kidneys following IR of the lower extremities.

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Key words: fullerenol C60, ischemia-reperfusion, sevoflurane, lower extremity, lung, kidney

Introduction

Ischemia-reperfusion (IR) injury is a complication that commonly occurs following medical and surgical interventions, such as thrombolytic therapy, organ transplantation, coronary angioplasty and cardiopulmonary bypass (1). The main issue surrounding IR injury is microvascular dysfunction after reperfusion of ischemic tissues, which subsequently leads to impaired endothelium-dependent dilatation in arterioles, increased fluid filtration, leukocyte occlusion in capillaries, leukocyte compression and plasma protein extravasation in post-capillary venules (2). Furthermore, activated endothelial cells in the microcirculation produce more oxygen radicals but less nitric oxide (NO) during the first period (5-20 min) after reperfusion and this imbalance between superoxide and NO in endothelial cells results in the production and release of inflammatory mediators, and increases the biosynthesis of adhesion molecules, thus mediating leukocyte-endothelial cell

adhesion (1-3). Inflammatory mediators released as a result of reperfusion may also activate endothelial cells in distant organs that were not initially exposed to IR injury (2,4). This distant response to IR can lead to leukocyte-dependent microvascular damage, which is characteristic of multiple organ dysfunction syndrome (1). Recently, it has been shown that reperfusion, a term used to describe blood flow restoration after ischemia, may place ischemic organs at a greater risk of cellular necrosis, thus limiting the return of function (5,6).

Halogenated inhalational anesthetics are currently the most common drugs used for the induction and maintenance of general anesthesia. Sevoflurane is a halogenated inhalation anesthetic widely used in general anesthesia (7), which has a positive effect on IR-induced lung injuries through the reduction in tumor necrosis factor-α (TNF-α) release (8).

There are numerous mechanisms that have not been elucidated in the prevention of IR injury, especially during and after lower extremity surgeries and the effectiveness of the currently used methods (such as ischemic preconditioning and antioxidant treatments) (9,10) is limited.

It has been shown in previous studies that sevoflurane administration is protective against IR injury; however, to the best of our knowledge, its effect alongside fullerenol C60, a nanoparticle, has not been determined (11,12). The activities of nanoparticles and their uses in nanomedicine have been subject to growing interest; thus, the present study aimed to investigate the protective effect of fullerenol C60 in rats treated with sevoflurane against damage to the lung and kidney tissues in lower extremity IR. The present study investigated the effects of fullerenol C60 and sevoflurane, alone or combined, on lung and kidney tissue in rats with lower extremity IR injury.

Materials and methods

Animals and experimental protocol. The present study was conducted at the Gazi University Animal Experiments Laboratory (Ankara, Turkey) in July 2021 in accordance with the ARRIVE guidelines (13). The study protocol was approved by the Animal Research Committee of Gazi University (G.Ü.ET-21.023). All of the animals were maintained in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (14).

Rats were anesthetized with ketamine [50 mg/kg, intraperitoneal (i.p.)] and Rompun® (20 mg/kg, i.p.) and placed on a heating pad to maintain their body temperature. Rats were kept in a temperature-controlled (21±1˚C) and humidity-controlled (45-55%) room, and were maintained under a 12-h light/dark cycle. The animals were fed a standard pellet diet and allowed to drink water *ad libitum*. The dose of ketamine and Rompun administered to the rats was in accordance with the study by Yesil *et al* (15).

A total of 30 Wistar albino male rats (Gazi University Animal Experiments Laboratory, Ankara, Turkey) (age, 8 months; weight, 225-275 g) were used in the present study. The animals were equally divided into the following five groups (n=6): i) Sham; ii) IR; iii) IR-fullerenol C60 (IR-FUL); iv) IR-sevoflurane (IR-SEVO); and v) IR-fullerenol C60-sevoflurane (IR-FUL-SEVO). The effects of sevoflurane (16) and fullerenol C60 (17) on normal rats have been investigated in previous studies and our ethics committee limited the number of rats to be used in the experiment due to the 4R rule (18); therefore, the groups of the present study were treated as follows: i) The sham group only underwent midline laparotomy without any additional surgical intervention; ii) the IR group was subjected to midline laparotomy and a traumatic microvascular clamp was placed in the infrarenal abdominal aorta for 120 min, after which it was removed and reperfused for 120 min. Sodium heparin (500 IU/kg) was administered through the peripheral tail vein to maintain reperfusion after occlusion; iii) the IR-FUL group underwent the same surgical procedures as the IR group with fullerenol C60 (Fullerene-C60; 98%; 1 g, CAS no. 99685-96-8; MilliporeSigma) administered (100 mg/kg, i.p) (19) 30 min before the ischemic period; iv) the IR-SEVO group underwent the same surgical procedures as the IR group. Anesthetic gas vaporizers were calibrated and set at a minimum alveolar concentration of sevoflurane (2.3%). Rats were anesthetized in a transparent plastic box (40x40x70 cm) and sevoflurane was administered at 2.3% inspiratory concentration at a rate of 4 l/min in 100% O_2 for 4 h; and v) the IR-FUL-SEVO group underwent the same surgical procedures as the IR group with fullerenol C60 administered 30 min before ischemia and sevoflurane administered throughout the IR period.

Following the end of the reperfusion period, all rats were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. injection, and were sacrificed by exsanguination during blood sample (5-10 ml) collection from the heart. After heart rate and respiration ceased, monitoring was continued for a further 2 min to confirm death. Then, lung and kidney tissues were removed for biochemical and histopathological analyses.

Biochemical analysis. Biochemical analyses were performed according to protocols used in our previous publications (20,21). Right lung and right kidney tissues were washed with cold NaCl solution (0.154 M) to remove blood contamination and then homogenized (Heidolph homogenizer DIAX 900; Heidolph Instruments GMBH & CO. KG) at 1,000 U for ~3 min. After centrifugation at 10,000 x g for ~10 min at 4˚C, the upper clear layer was collected for analysis.

The thiobarbituric acid reactive substances (TBARS) assay was performed according to the protocol described by Van Ye *et al* (22). Catalase (CAT) activity was measured using methods described by Aebi (23) and glutathione S-transferase (GST) enzyme activity was measured according to methods described by Habig *et al* (24). The amount of sample protein was determined using the Lowry method with BSA (MilliporeSigma) used as the standard protein (25). The results were expressed as IU/mg protein for enzymes and nmol/mg protein for TBARS.

Histopathological assessment of kidney and lung tissue specimens. Tissue samples taken from the periphery of the left lung and left kidney were fixed in 10% neutral buffered formalin for 72 h at room temperature. Following fixation, tissue samples were processed using an increasing grade alcohol series, cleared in xylene and embedded in paraffin blocks. Kidney and lung sections (4 μm) were obtained using a Leica RM2245 rotary microtome (Leica Microsystems GmbH). Thereafter, sections were deparaffinized in xylene and rehydrated through decreasing grade alcohol series, and

placed in distilled water to prepare them for histochemical and immunohistochemical staining, and TUNEL assay.

Lung and kidney sections were stained with hematoxylin for 12 min at room temperature and eosin for 12 min at room temperature. Kidney sections were also stained with Periodic acid-Schiff (PAS). To that end, sections were incubated in Periodic acid solution and Schiff reagent at room temperature in dark for 35 and 40 min, respectively; and immersed in hematoxylin for nuclear staining for 1 min at room temperature. The stained sections were observed under a Leica DM4000 B light microscope (Leica Microsystems GmbH) equipped with a computer and images were captured using Leica LAS v4.9 (Leica Microsystems GmbH).

Hematoxylin and eosin (H&E)- and PAS-stained kidney samples were examined under x200 and x400 magnifications and renal injury was assessed semi-quantitatively. Swelling, vacuolization and loss of brush borders in tubular epithelial cells, as well as epithelial cell sloughing and hyaline cast formation were considered indicators of kidney injury and were evaluated in 10 randomly chosen fields from the cortex of each kidney section. A scoring system for the ratio of injured tubules displaying the aforementioned findings was applied as follows: 0, no tubular injury; 1, ≤10% of tubules; 2, 10-25% of tubules; 3, 25-45% of tubules; 4, 45-75% of tubules; and 5, >75% of tubules were involved in injury. The average score for kidney injury was calculated for each kidney sample (26,27).

Lung injury was evaluated using the lung injury scoring system developed by The American Thoracic Society (28). For this purpose, 20 non-overlapping fields of H&E-stained lung sections were examined under x200 and x400 magnifications and the following parameters were scored for each field: (A) Neutrophils in the alveolar space (0, none; 1, 1-5; 2, >5); (B) neutrophils in the interstitial space (0, none; 1, 1-5; 2, >5); (C) hyaline membranes (0, none; 1, 1; 2, >1); (D) proteinaceous debris filling the airspaces (0, none; 1, 1; 2, >1); and (E) alveolar septal thickening (0, <2x; 1, 2x-4x; 2, >4x). The sum of the injury scores for each animal was determined using the following formula: Score=[(20 x A) + (14 x B) + (7 x C) + (7 x D) + (2 x E)]/(no. of fields x100) (28,29). Tubular injury scores and lung injury scores were compared between the groups.

Immunohistochemical assessment of kidney and lung tissue specimens. For immunostaining of tissue sections, deparaffinization and rehydration were followed by heat-induced antigen retrieval in citrate buffer (pH 6.0) for 2 h in a water bath adjusted to 85˚C. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 30 min in dark at room temperature. To perform the protein blocking to prevent nonspecific binding of antibodies sections were incubated with Ultra V Block solution (cat. no. TA-125-UB; Thermo Fisher Scientific, Inc.) for 30 min at room temperature, kidney and lung tissue sections were incubated with anti-tumor necrosis factor-α (TNF-α; 1:100; Elabscience Biotechnology, Inc.; cat. no. E-AB-33121), anti-interleukin 1β (IL-1β; 1:100; Elabscience Biotechnology, Inc.; cat. no. E-AB-66749) and anti-intercellular adhesion molecule 1 (ICAM-1; 1:100; BIOSS; cat. no. bs-0608R) primary antibodies to investigate the inflammatory processes. Additionally, kidney sections were incubated with anti-B-cell lymphoma 2 (BCL-2)-associated X protein (BAX; 1:100; BIOSS; cat. no. bs-0127R), anti-BCL-2 (1:200; BIOSS; cat. no. bs-4563R) and anti-caspase-3 (CASP-3; 1:100; Elabscience Biotechnology, Inc.; cat. no. E-AB-66940) primary antibodies to examine the apoptotic processes. Incubation with primary antibodies overnight at 4˚C was followed by incubation with biotinylated secondary antibody (cat. no. TP-125-BN; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. Afterward, HRP-labeled streptavidin (cat. no. TS-125-HR; Thermo Fisher Scientific, Inc.) was applied to sections for 30 min in dark at room temperature. The staining procedure was completed using the 3,3'-diaminobenzidine (DAB) chromogen. For the assessment of stained sections, 10 randomly chosen, non-overlapping fields were captured under x400 magnification using a Leica DM4000 B light microscope (Leica Microsystems GmbH) and immunopositive staining intensity was semi-quantified using ImageJ software (1.48v; National Institutes of Health) and expressed as % area (30).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for kidney samples. The processes before the TUNEL assay were the same as those performed before histological analysis; the kidney samples were fixed in 10% neutral buffered formalin for 72 h at room temperature, and then processed for paraffin embedding. Tissue sections (4 μm) were deparaffinized and rehydrated prior to the TUNEL assay. Apoptotic epithelial cells of the kidney tubules were determined using a TUNEL assay kit (Elabscience Biotechnology, Inc.; cat. no. E-CK-A331-50T); the assay was performed in accordance with the manufacturer's protocol and sections were mounted with anhydrous mounting medium Entellan™ new (cat. no. 107961; MilliporeSigma). Tubular epithelial cells with brown nuclei stained with DAB (0.5 mg/ml) for 5 min at room temperature were considered $TUNEL^+$ cells. Apoptotic cells were counted in 10 randomly chosen fields under x400 magnification using the Leica DM4000 B light microscope (Leica Microsystems GmbH) and the results were expressed as number of $TUNEL^+$ cells per field (31).

Statistical analysis. All data are expressed as the mean ± standard deviation. All statistical analyses were performed using SPSS (version 20.0; IBM Corp.). The distribution of data was analyzed using the Shapiro-Wilk test. Comparisons among >2 groups were carried out using the Kruskal-Wallis test followed by Dunn's test or one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histopathological findings. In contrast to the almost normal appearance, with slight congestion, of the lung tissue sections from the sham group, marked congestion and neutrophil infiltration were observed in the lung samples of animals from the IR group. While neutrophil infiltration in the alveolar spaces of the lung specimens from animals in the IR group were more prominent, there was infiltration in the interstitial space in the treatment groups, which was accompanied by alveolar septum thickening observed in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups (Fig. 1). It was found that IR significantly increased the lung injury score compared with the sham group. Furthermore, lung injury scores were significantly lower

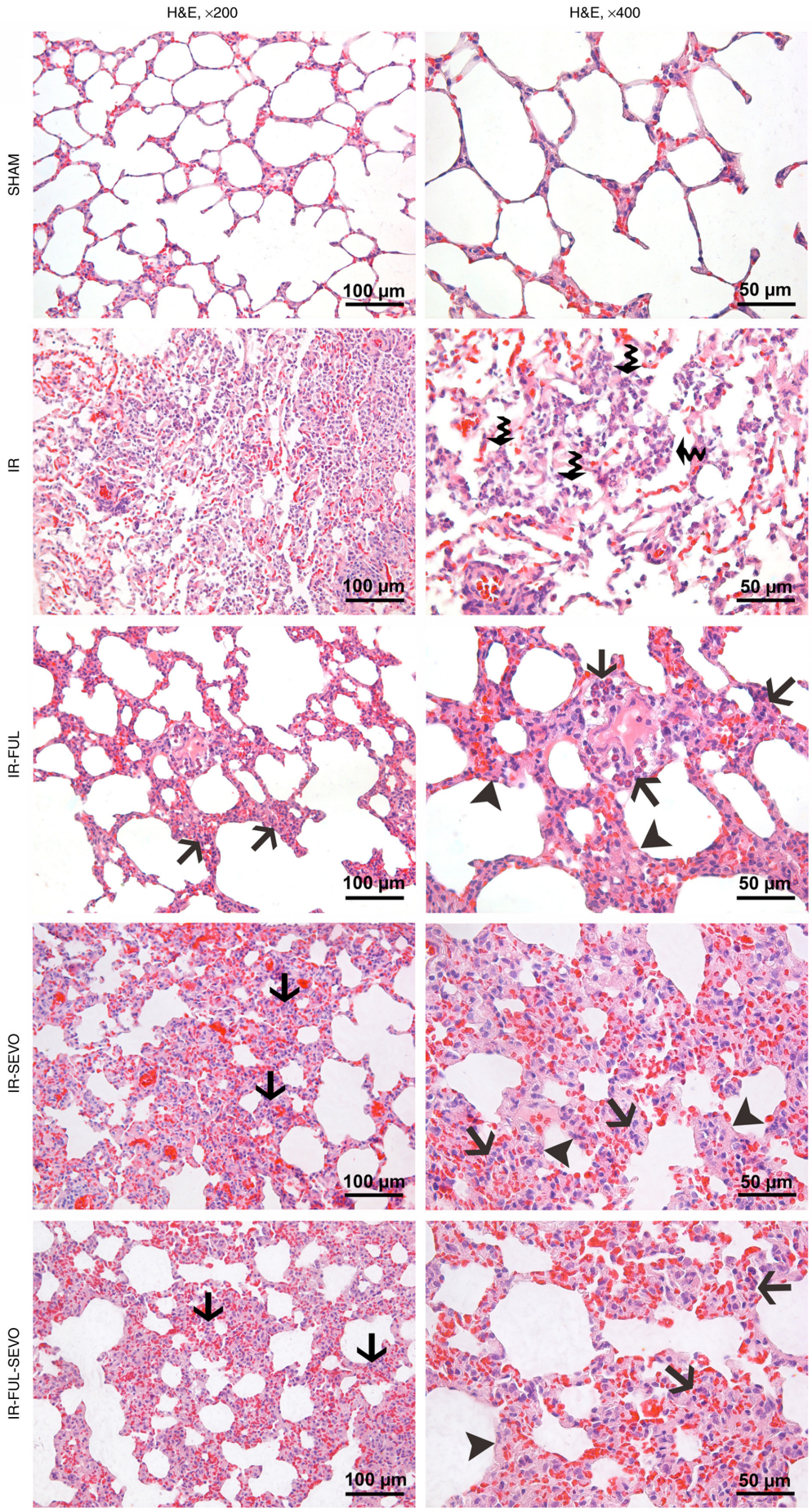

Figure 1. H&E-stained lung sections. The 'arrow head' indicates alveolar septal thickening, the 'arrow' indicates neutrophils in the interstitial space and the 'waved arrow' indicates neutrophils in the alveolar space. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane; H&E, hematoxylin and eosin.

Table I. Lung and kidney tubule injury scores [median (IQR)].

Variable	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	Kruskal-Wallis P-value
Lung injury score	0.020 (0.016-0.024)	0.25 (0.22-0.28)[a]	0.19 (0.11-0.22)[a,b]	0.20 (0.16-0.23)[a]	0.17 (0.15-0.22)[a,b]	<0.0001
Kidney tubules injury score	0.35 (0.17-0.60)	4.40 (4.15-4.62)[a]	3.05 (2.75-3.50)[a]	4.40 (3.87-4.50)[a]	3.85 (3.40-3.85)[a]	<0.0001

P-values were calculated with Kruskal-Wallis test. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

Table II. Comparison for the immunostaining intensity of lung samples labelled with TNF-α, IL-1β and ICAM-1 antibodies (mean ± SD).

Protein	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	ANOVA P-value
TNF-α	1.91±0.48	9.43±2.03[a]	2.84±0.59[b]	4.89±1.74[a-c]	3.86±1.05[a,b]	<0.0001
IL-1β	2.23±0.56	9.63±1.96[a]	3.69±0.61[b]	7.45±1.45[a,c]	4.67±0.72[a,b]	<0.0001
ICAM-1	1.66±0.54	21.96±8.50[a]	4.87±0.86[b]	14.34±3.06[a-c]	9.10±3.42[a,b]	<0.0001

P-values were calculated with one-way ANOVA. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group, [c]P<0.005 compared with the IR-FUL group. TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; ICAM-1, intercellular adhesion molecule 1; IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

Table III. Comparison for immunostaining intensity of kidney samples labelled with TNF-α, IL-1β and ICAM-1 antibodies (mean ± SD).

Protein	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	ANOVA P-value
TNF-α	3.75±1.54	23.88±3.36[a]	10.36±2.60[a,b]	16.49±2.92[a-c]	12.84±3.27[a,b]	<0.0001
IL-1β	12.78±3.22	23.28±22.12[a]	17.63±1.21[a,b]	20.91±1.59[a-c]	19.60±2.28[a,b]	<0.0001
ICAM-1	10.79±3.41	25.77±3.01[a]	18.66±1.03[a,b]	22.59±1.15[a-c]	20.69±1.12[a,b]	<0.0001

P-values were calculated with one-way ANOVA test. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group, [c]P<0.005 compared with the IR-FUL group. TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; ICAM-1, intercellular adhesion molecule 1; IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

in the IR-FUL and IR-FUL-SEVO groups compared with the IR group (P=0.004 and P=0.017, respectively; Table I). In the histopathological evaluation of kidney samples, varying degrees of tubular injury, ranging from vacuolization, loss of brush border in tubular epithelial cells to loss of tubular epithelium and hyaline cast formation, were observed in all IR groups (Fig. 2). There was an evident increase in the mean kidney tubule injury score in all IR groups compared with the sham group (P<0.0001). Additionally, the mean kidney injury score was significantly lower in the IR-FUL and IR-FUL-SEVO groups compared with that in the IR group (P<0.0001 and P=0.014, respectively) (Fig. 2; Table I).

Immunohistochemical findings. The lung tissue expression of TNF-α, IL-1β and ICAM-1 increased significantly following hindlimb IR, whereas the expression of these markers decreased significantly with the use of fullerenol C60. When the improvement was compared between the treatment groups, the effect of fullerenol C60 alone was greater than when compared with sevoflurane alone (Table II; Fig. 3).

When the kidney tissue was examined for TNF-α, IL-1β and ICAM-1 expression following hindlimb IR injury, it was observed that elevated expression levels of these markers were significantly improved by the administration of fullerenol 60 alone and this effect was more prominent than that of sevoflurane alone (Table III; Fig. 4).

A significant elevation in BAX and CASP-3 expression, and a significant reduction in BCL-2 expression was detected in kidney samples following hindlimb IR injury. These alterations improved considerably in all treatment groups, although the best outcomes were observed in the group treated with fullerenol C60 alone (Table IV; Fig. 5). Similarly, the

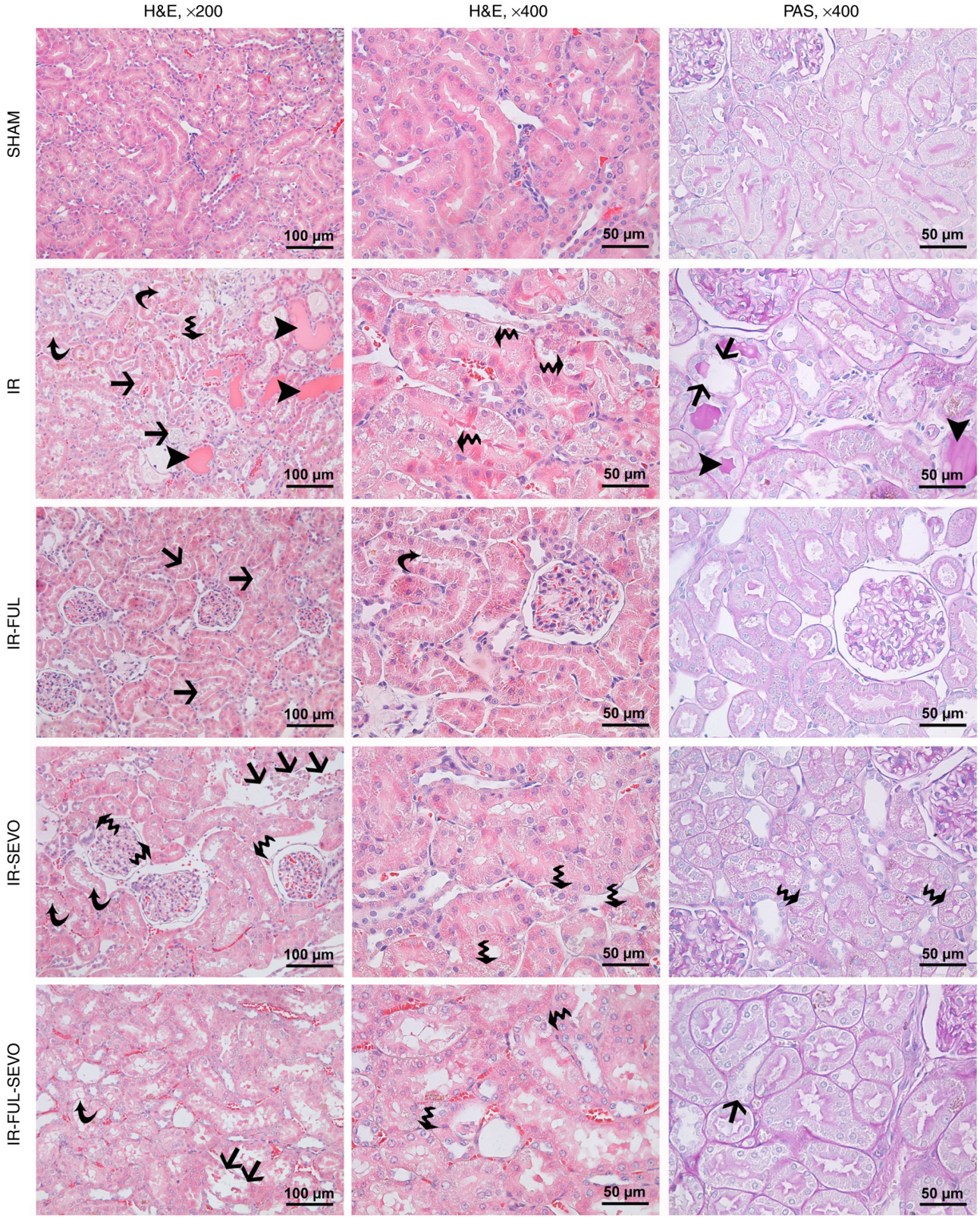

Figure 2. H&E- and PAS-stained kidney sections. The 'arrow head' indicates hyaline cast formation, the 'arrow' indicates varying degrees of tubular epithelium loss, the 'waved arrow' indicates vacuolization in tubular epithelial cells and the 'curved arrow' indicates a loss of brush border in the tubule epithelial cells. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane; H&E, hematoxylin and eosin; PAS, Periodic acid-Schiff.

TUNEL assay revealed a considerable increase in the number of tubular cells undergoing apoptosis following hindlimb IR injury, whereas a significant amelioration was achieved in all treatment groups (Table IV; Fig. 5).

Biochemical findings. When the lung tissue TBARS levels were compared between the groups, a significant difference was observed (P<0.0001). In the IR and IR-SEVO groups, TBARS levels were considerably higher than those

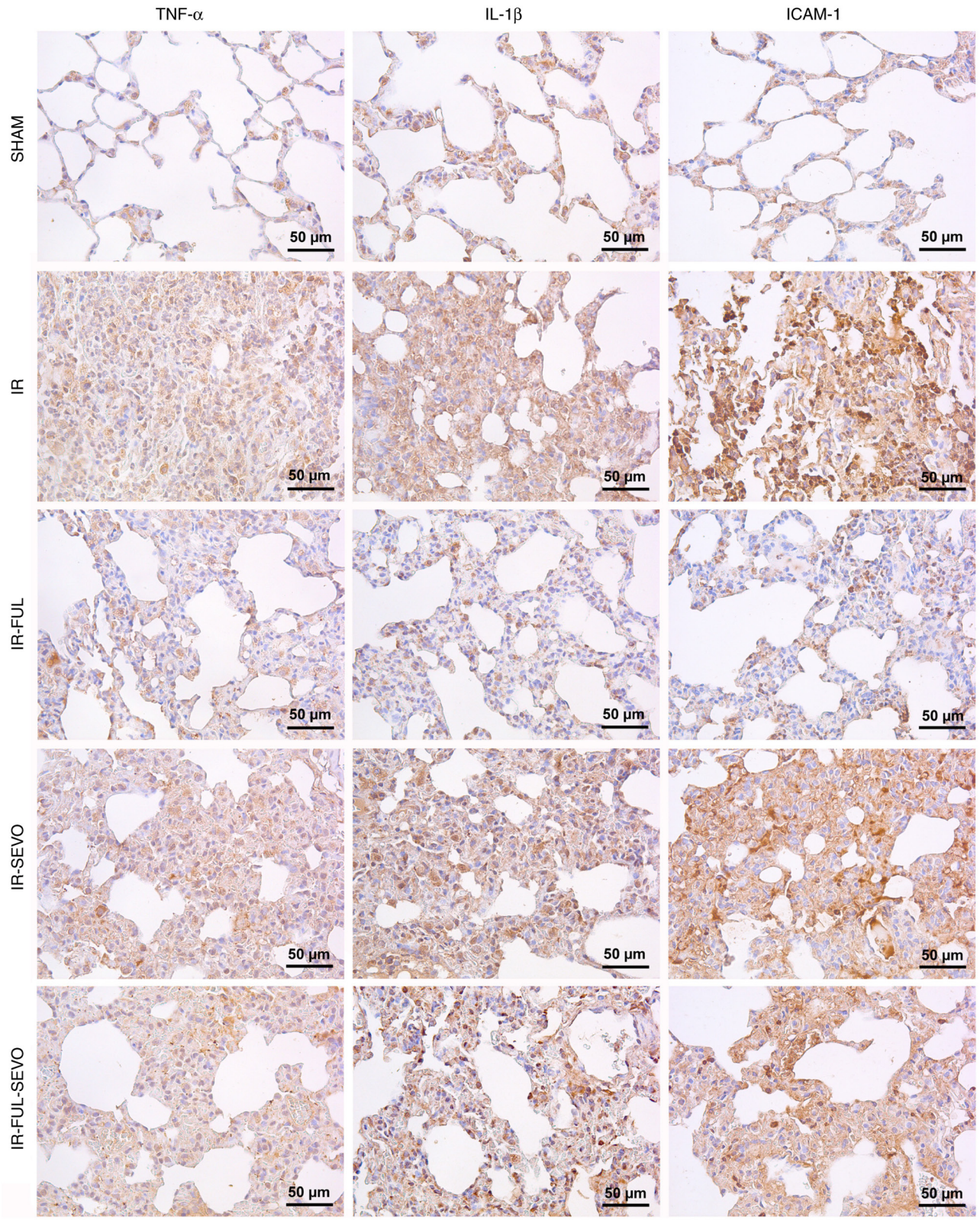

Figure 3. Representative micrographs of lung sections labelled with antibodies against TNF-α, IL-1β and ICAM-1. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; ICAM-1, intercellular adhesion molecule 1.

in the sham group (P<0.0001 and P=0.007, respectively). Additionally, the TBARS levels in the IR-SEVO group were considerably higher than those in the IR-FUL group (P=0.031). TBARS levels were significantly lower in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups compared with those in the IR group (P<0.0001, P=0.017 and P=0.001, respectively; Table V).

A significant difference was found between the groups in terms of CAT enzyme activity in lung tissue (P<0.0001). CAT enzyme activity was significantly decreased in the IR,

Table IV. Comparison for immunostaining intensity of kidney samples labelled with BAX, BCL-2 and CASP-3 antibodies, and TUNEL positivity in renal tubular cells (mean ± SD).

Variable	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	ANOVA P-value
BAX	2.68±0.71	7.02±1.67[a]	3.76±0.76[b]	5.47±1.41[a-c]	4.52±1.02[a,b]	<0.0001
BCL-2	7.41±1.15	2.80±0.64[a]	6.26±1.84[b]	4.70±1.53[a,b]	5.70±1.48[b]	<0.0001
CASP-3	20.14±1.18	29.46±3.22[a]	22.93±0.27[a,b]	24.98±1.28[a-c]	23.44±0.95[a,b]	<0.0001
TUNEL	0.05±0.02	3.33±0.53[a]	0.11±0.04[b]	0.57±0.33[b]	0.32±0.26[b]	<0.0001

P-values were calculated with one-way ANOVA. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group, [c]P<0.005 compared with the IR-FUL group. BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; CASP3, caspase-3; IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

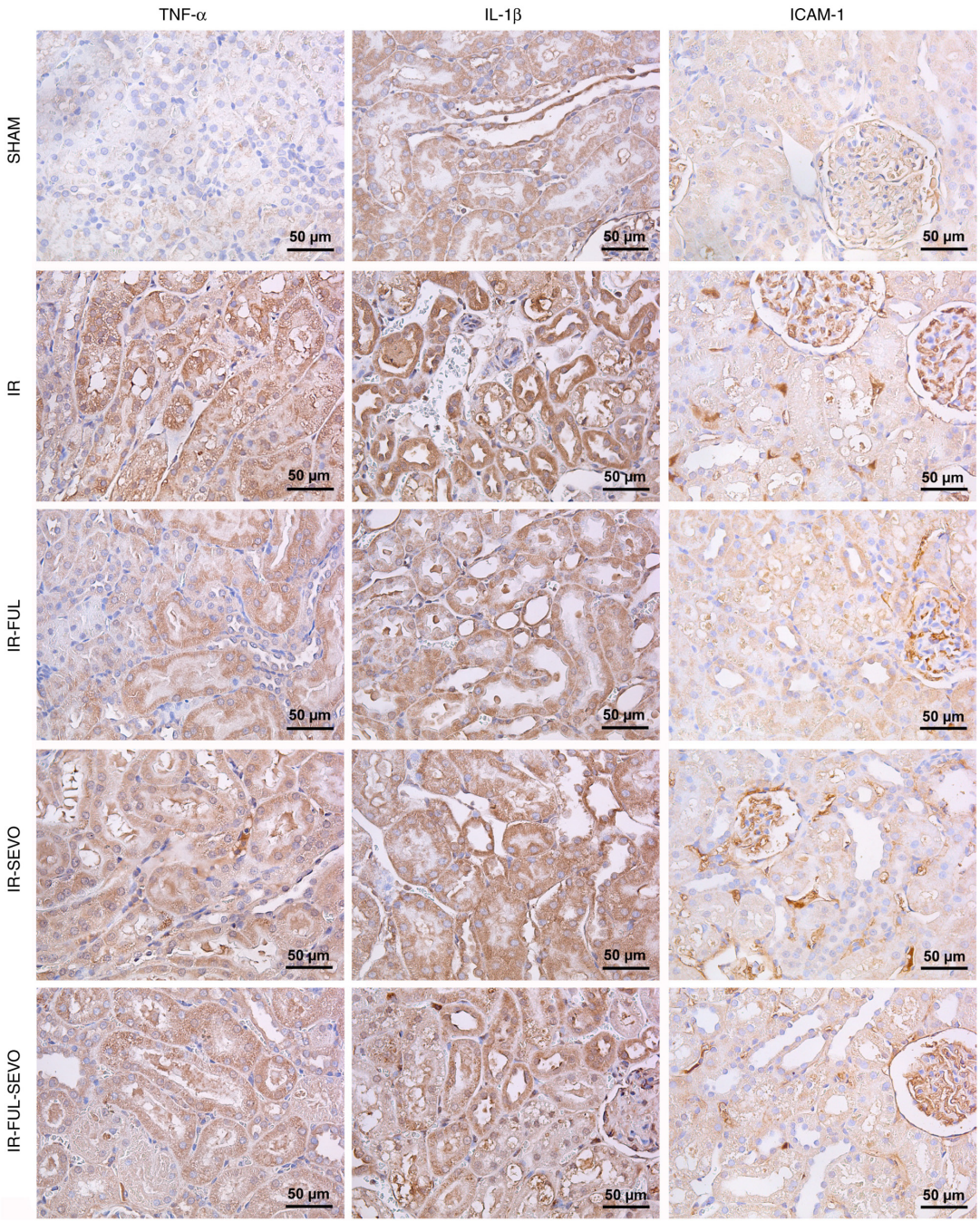

Figure 4. Representative micrographs of kidney sections labelled with antibodies against TNF-α, IL-1β and ICAM-1. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; ICAM-1, intercellular adhesion molecule 1.

Table V. Biochemical data of lung tissue (mean ± SD).

Variable	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	ANOVA P-value
TBARS (nmol/mg.pro)	0.43±0.07	1.04±0.10[a]	0.50±0.09[b]	0.76±0.05[a-c]	0.63±0.10[b]	<0.0001
CAT (IU/mg.pro)	60.25±3.48	25.32±1.76[a]	56.58±3.49[b]	43.83±5.41[a-c]	47.32±4.18[a,b]	<0.0001
GST (IU/mg.pro)	0.90±0.14	0.28±0.04[a]	0.77±0.12[b]	0.66±0.07[a,b]	0.82±0.06[b]	<0.0001

P-values were calculated with one-way ANOVA. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group, [c]P<0.005 compared with the IR-FUL group. TBARS, thiobarbituric acid reactive substances; CAT, catalase; GST, glutathione S-transferase; IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

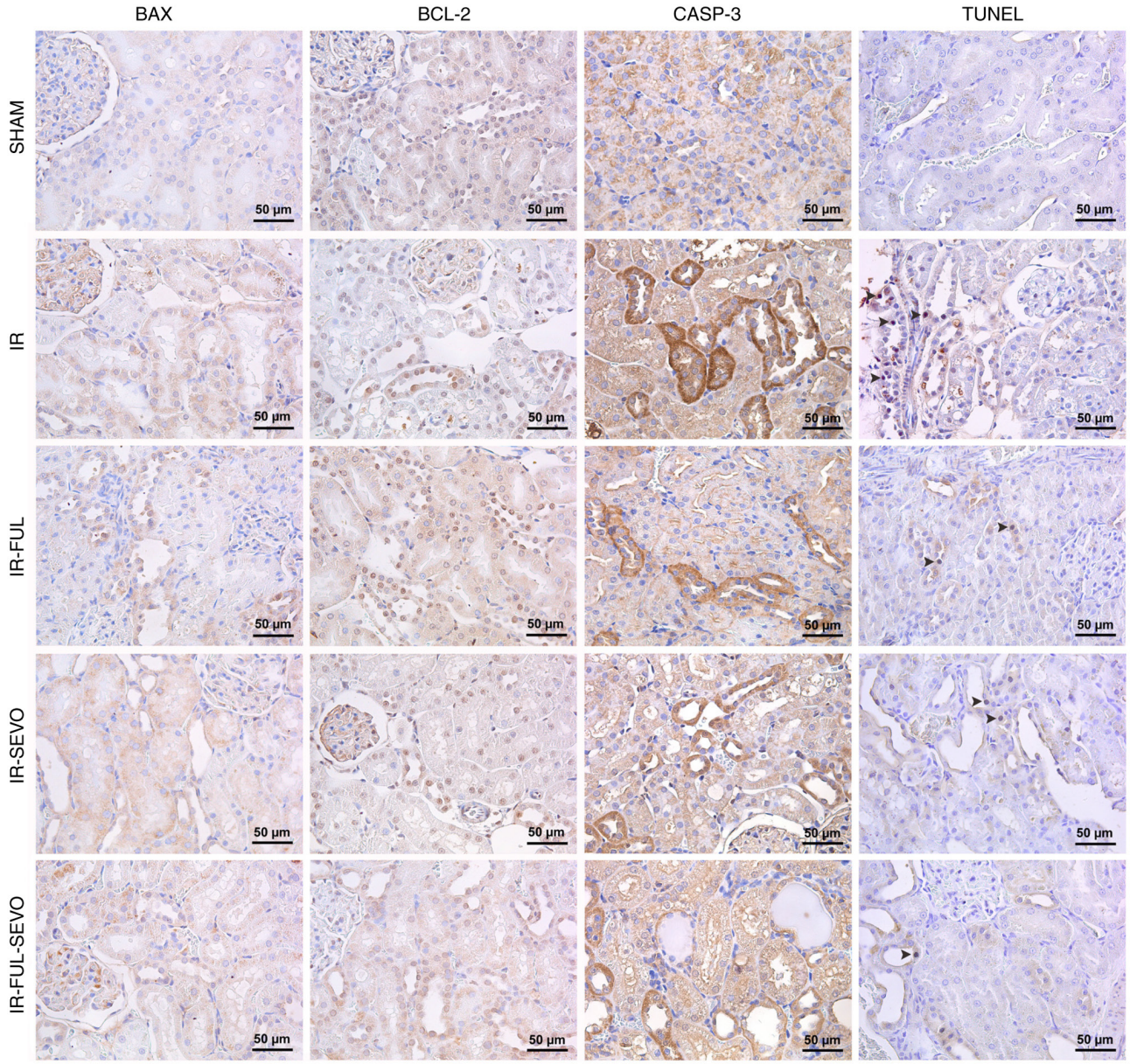

Figure 5. Representative micrographs of kidney sections demonstrating TUNEL positivity and expression of apoptosis-related markers BAX, BCL-2 and CASP-3. The 'arrow head' indicates TUNEL$^+$ cell nuclei. IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane; BCL-2, B-cell lymphoma 2; BAX, BCL-2-associated X protein; CASP3, caspase-3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

IR-SEVO and IR-FUL-SEVO groups compared with that in the sham group (P<0.0001, P=0.006 and P=0.025, respectively). Additionally, CAT activity was significantly lower in the IR-SEVO group than in the IR-FUL group (P=0.027). CAT

Table VI. Biochemical data of kidney tissue (mean ± SD).

Variable	Sham (n=6)	IR (n=6)	IR-FUL (n=6)	IR-SEVO (n=6)	IR-FUL-SEVO (n=6)	ANOVA P-value
TBARS (nmol/mg.pro)	0.83±0.10	1.83±0.14[a]	1.01±0.12[b]	1.46±0.09[a-c]	1.11±0.10[b]	<0.0001
CAT (IU/mg.pro)	70.47±3.62	44.28±2.10[a]	63.58±4.34[b]	54.65±2.30[a,b]	57.98±4.18[a,b]	<0.0001
GST (IU/mg.pro)	1.75±0.10	0.78±0.06[a]	1.74±0.15[b]	1.37±0.13[a-c]	1.50±0.09[b]	<0.0001

P-values were calculated with one-way ANOVA. [a]P<0.05 compared with the sham group, [b]P<0.005 compared with the IR group, [c]P<0.005 compared with the IR-FUL group. TBARS, thiobarbituric acid reactive substances; CAT, catalase; GST, glutathione S-transferase; IR, ischemia-reperfusion; FUL, fullerenol C60; SEVO, sevoflurane.

enzyme activity was significantly higher in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups than in the IR group (P<0.0001, P=0.002, P<0.0001, respectively; Table V).

There was a significant difference between the groups in terms of GST activity in lung tissue (P<0.0001). GST activity was significantly lower in the IR and IR-SEVO groups than in the sham group (P<0.0001 and P=0.048, respectively). GST enzyme activity was significantly higher in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups than in the IR group (P=0.001, P=0.010, P<0.0001, respectively; Table V).

In kidney tissue, TBARS levels were considerably higher in the IR and IR-SEVO groups, than those in the sham group (P<0.0001 and P<0.0001, respectively). Also, it was considerably higher in the IR-SEVO group compared with that in the IR-FUL group (P=0.004). However, TBARS levels were significantly lower in IR-FUL, IR-SEVO and IR-FUL-SEVO groups compared with those in the IR group (P<0.0001, P=0.016 and P<0.0001, respectively; Table VI).

CAT enzyme activity was significantly decreased in the IR, IR-SEVO and IR-FUL-SEVO groups compared with that in the sham group (P<0.0001, P=0.003 and P=0.016, respectively). CAT enzyme activity was significantly higher in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups compared to the IR group (P=0.001, P=0.043, P=0.009, respectively) (Table VI).

GST activity was significantly lower in the IR and IR-SEVO groups compared with the sham group (P<0.0001 and P=0.018, respectively). Additionally, GST activity was significantly lower in the IR-SEVO group compared with that in the IR-FUL group (P=0.019). GST enzyme activity was significantly higher in the IR-FUL, IR-SEVO and IR-FUL-SEVO groups compared with that in the IR group (all P<0.0001) (Table VI).

Discussion

During IR, serious damage occurs to tissues with inflammatory mediators released during the reperfusion period leading to damage by activating endothelial cells in distant organs (4). Furthermore, this response to IR can lead to leukocyte-dependent microvascular damage (4). Restoring blood flow in the ischemic limb may save the limb; however, multisystem organ failure may develop, which can be fatal (10,32). The findings of the present study support the idea that oxidative damage due to ischemia of the lower extremity results in damage to lung and kidney tissue with reperfusion. However, fullerenol C60 administered 30 min before ischemia and sevoflurane administered during IR were observed to be protective against this damage.

Restoring the blood flow in the ischemic limb may save the limb; however, multisystem organ failure may develop, which can be fatal (33). The severity of the inflammatory response in tissues after ischemia may be similar in distant organs. Pulmonary vasoconstriction and respiratory dysfunction have been shown to occur in humans after aortic replacement, independent of capillary wedge pressure, following aortic clamping and reperfusion of the lower extremities (34). IR in the lower extremities may cause pulmonary damage and dysfunction, characterized by interstitial edema, requiring prolonged ventilatory and inotropic support in some patients (10,32). Surgery of the infrarenal aorta and the great arteries of the lower extremities may cause rhabdomyolysis in the skeletal muscle, which may result in remote kidney damage (35).

A number of studies have shown that volatile anesthetics are protective against IR damage by reducing inflammation (36-38). It has been proven that the administration of sevoflurane protects distant organs, such as the heart (39), lungs (40) and kidneys (36) against IR damage. Lee *et al* (36) examined the protective effects of volatile anesthetics against IR damage, but it was not clear at what stage they were effective. By contrast, in a previous study, 42 patients [classified as American Society of Anesthesiologists physical status 1 (40 men, 2 women] who were scheduled to undergo dental or orthopedic surgery that was expected to last ≥4 h were studied. Patients who showed evidence of abnormal hepatic or renal function, based on medical history, physical examination or laboratory tests, were excluded from the study. The results revealed that low-flow sevoflurane anesthesia may cause proteinuria; however, the observed proteinuria was not associated with any changes in blood urea nitrogen, creatinine or creatinine clearance in patients without pre-existing kidney disease (41). In the present study, the protective effect of sevoflurane against IR damage was supported by both histopathological and biochemical data.

Fullerenol, a new nanoparticle, is used in medicine, as well as in numerous branches of science. Owing to its spherical molecules with 30 carbon double bonds, fullerenol

C60 can easily react with free radicals, thus acting as an effective free radical scavenger that can be labeled a 'radical sponge' (42). Chen *et al* (43) showed that fullerenol C60 has antioxidant activities at low concentrations and protects the lung tissue from IR injury. Since the cytotoxic effect of high concentration fullerenol C60 was proven in a previous study, low concentrations of fullerenol C60 were used in the present study (the maximum dose of fullerenol C60 was 100 μg/ml to avoid cytotoxicity) (44,45). As aforementioned, fullerenes exhibit strong antioxidant effects. It has been proven by studies that they are protective against renal IR injury induced by oxidative stress through this mechanism (46,47). In addition, in a study using fullerenol C60 in IR injury of skeletal muscle, it was determined that both intramuscularly and intravenously administered fullerene treated ischemic pathologies without showing a cytotoxic effect (48). In another study, it was revealed that fullerenol C60 ameliorated ischemic renal failure by reducing the formation of apoptosis; pretreatment with fullerenol C60 into the kidneys diminished apoptosis (as determined by TUNEL staining, the detection of apoptotic particles, and assesment of BCL-xl mRNA and protein expression), and DNA laddering (49). In the present study, kidney damage score was significantly lower in the group receiving fullerenol C60 compared with the IR group.

The present study focused on the beneficial effects of fullerenol C60 administration with sevoflurane; however, there were a number of limitations. Firstly, theoretically, a single dose of fullerenol C60 may not be sufficient to prevent lower extremity IR injury (50). The effects of IR injury persist over a long period of time; however, the animal models in the present study were euthanized at the end of the experiment (51). It would have been beneficial to include rats that were euthanized at different time points after fullerenol C60 administration and thus, this requires further investigation. In addition, only a single concentration of fullerenol C60 was used in the present study. Finally, the results of the present study should have been supported by experimental methods, such as western blotting; however, due to funding restrictions, this was not feasible. Future studies will include these methods to further validate the results.

In conclusion, the present study demonstrated the effectiveness of fullerenol C60 in the lung and kidney tissues of rats under sevoflurane anesthesia after lower extremity IR through reduction of oxidative and histopathological damage in the lungs and kidneys. Reducing the oxidative damage associated with IR has become a target for drug studies in this area and a number of molecules (albumin nanoparticles, PLGA nanoparticles, exosomes, chitosan nanoparticles, polymeric micelles) that inhibit oxidative stress have been previously investigated (52-54). The results of the present study indicated that fullerenol C60 may be considered as a potential inhibitor of lower-extremity IR injury and, to the best of our knowledge, the present study was the first to investigate the effects of fullerenol C60 on distant organ damage in a lower-extremity IR model under sevoflurane anesthesia. Therefore, considering the limitations of the present study, future studies with additional methods of analysis would positively contribute to the literature and support these results.

Acknowledgements

Not applicable.

Funding

This study was supported by the Gazi University BAP coordination unit within the scope of the project numbered TGA-2021-7231.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MA, AHA and AK designed the study, and analyzed and interpreted data. VŞ, MA and ZK performed the experiments. MA, AHA and ZK confirm the authenticity of all the raw data. ZK, AK, MA and ZY provided scientific and technical assistance, and critically revised the article for important intellectual content. VŞ and MA collected samples. ZY, SÖAD and MK performed cellular and molecular experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for the study was obtained from Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-21.023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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