Life Sciences 232 (2019) 116634

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Oleuropein suppresses oxidative, inflammatory, and apoptotic responses following glycerol-induced acute kidney injury in rats

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ARTICLE INFO

Keywords:
Oleuropein
Rhabdomyolysis
Acute kidney injury
Oxidative stress
Inflammation
Apoptosis

ABSTRACT

Aim: Here, we evaluated the possible protective effects of oleuropein, the major phenolic constituent in virgin olive oil against glycerol-induced acute kidney injury (AKI) in rats.

Main methods: Twenty-eight Sprague Dawley rats were allocated equally into four groups as follows: control group, oleuropein group (50 mg/kg body weight), AKI group and the oleuropein + AKI group. AKI was induced by injecting 50% glycerol (10 ml/kg body weight) intramuscularly.

Key findings: Glycerol injection increased the kidney relative weight as well as rhabdomyolysis (RM)- and AKI-related index levels, including the levels of creatine kinase, lactate dehydrogenase, creatinine, urea, and Kim-1 expression. Additionally, alteration in oxidative conditions in renal tissue was recorded, as confirmed by the elevated malondialdehyde and nitric oxide levels and the decreased glutathione content. Concomitantly, the protein and mRNA expression levels of antioxidant enzymes were suppressed. Moreover, *Nfe2l2* and *Hmox1* mRNA expression was also downregulated. Glycerol triggered inflammatory reactions in renal tissue, as evidenced by the increased pro-inflammatory cytokines and Ccl2 protein and mRNA expression, whereas myeloperoxidase activity was increased. Furthermore, glycerol injection enhanced apoptotic events in renal tissue by increasing the expression of the pro-apoptotic proteins and decreasing that of anti-apoptotic. However, oleuropein administration reversed the molecular, biochemical, and histological alterations resulting from glycerol injection.

Significance: Our data suggest that oleuropein has potential as an alternative therapy to prevent or minimize RM incidence and subsequent development of AKI, possibly due to its potent anti-stress, anti-inflammatory, and anti-apoptotic effects.

1. Introduction

Acute kidney injury (AKI) is a heterogeneous condition resulting from the impairment of kidney function that leads to changes in fluid volume, electrolyte levels, and retention of waste products. Acute kidney injury is predominantly diagnosed through elevated levels of serum creatinine and/or decreased urine outflow [1]. Uncontrolled AKI is strongly associated with chronic kidney injury progression, which further leads to death [2]. The main causes of AKI include renal hypoperfusion, renal tissue deformation, and urinary outflow obstruction [3]. Additionally, several diseases have been recognized as risk factors for AKI development, including diabetes and cardiovascular, liver, and pulmonary diseases [4]. Moreover, AKI incidence has been linked with rhabdomyolysis (RM), which is characterized by myocyte damage that results in the leakage of intracellular contents into the circulation. These contents include lactate dehydrogenase, myoglobin, glutamic

Abbreviations: AKI, acute kidney injury; Bax, BCL2-associated X protein; Bcl-2, *B-cell lymphoma 2*; CAT, catalase; CCL2, chemokine (C−C motif) ligand 2; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HO-1, heme oxygenase-1; IL-1β, interleukin 1-beta; IL-2, interleukin 2; iNOS, inducible nitric oxide synthase; Kim-1, kidney injury molecule-1; MPO, myeloperoxidase; NO, nitric oxide; Nrf2, nuclear factor erythroid 2–related factor 2; RM, Rhabdomyolysis; SD, Sprague Dawley; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha

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https://doi.org/10.1016/j.lfs.2019.116634
Received 3 May 2019; Received in revised form 30 June 2019; Accepted 3 July 2019
Available online 04 July 2019
0024-3205/ Published by Elsevier Inc.

oxalacetic transaminases, creatine kinase, glutamic oxalacetic transaminase, aldolase, and electrolytes [5]. Rhabdomyolysis is associated with numerous diseases, medications, injuries, and nephrotoxic molecules [6]. Rhabdomyolysis-induced AKI represents 10 to 40% of all diagnosed AKI cases [7]. Glycerol-induced RM, and subsequently AKI, is an accepted model used to understand the pathogenic mechanisms of AKI [8]. This experimental model is characterized by the excessive release of myoglobin, tubular obstruction, and renal vasoconstriction [9]. Although the exact mechanisms involved in RM-related AKI are unclear, several factors are suggested to play primary roles, including disruption of the cellular detoxifying system, programmed cell death activation, and inflammation of kidney tissue [5].

Antioxidant administration has been found to minimize, and protect against, myoglobinuria-induced AKI in renal tissue [6,8]. Olive tree products have been widely used in nutrition and for medicinal purposes for centuries. The therapeutic properties of the olive tree are due to its high antioxidant polyphenolic content [10]. Oleuropein is the most prevalent polyphenolic compound in all the parts of the olive tree, representing up to 14% of the dry weight, and is responsible for the characteristic bitter taste of unprocessed olives [11]. Oleuropein exhibits numerous biological and pharmacological activities, including antioxidant [12], anti-inflammatory [13], antiapoptotic [14], neuroprotective [15], and renoprotective [16]. Currently, no specific therapy has been approved for the prevention or treatment of AKI [2], making it necessary that nephroprotective agents with minimal side effects are identified. Therefore, this study was carried out to investigate the potential renoprotective effect of oleuropein against glycerol-induced renal impairments by estimating the kidney index, markers of renal function, oxidative challenge, inflammatory signaling, expression of apoptotic proteins, and histopathological deformations in the renal tissue of the rat.

2. Materials and methods

2.1. Materials and chemicals

Glycerol and oleuropein were sourced from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents in the present investigation were of analytical grade.(See Fig. 1.)

2.2. Experimental design

Twenty-eight SD rats (180–200 g; 13 weeks old) were housed in polypropylene cages, in the animal facility of the Zoology and Entomology Department, Faculty of Science, Helwan University (Cairo, Egypt) under controlled temperature (24–26 °C) and an artificial 12-h light/dark cycle. All experiments were performed under anesthesia and all efforts were made to minimize suffering. All experimental protocols, including the use of animals, were approved by the Committee of Research Ethics for Laboratory Animal Care, Department of Zoology and Entomology, Faculty of Science, Helwan University (approval no, HU2018/Z/08), in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, 8th edition (NIH Publication no. 85–23, revised 1985).

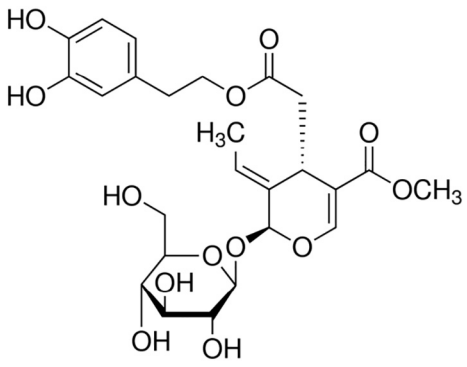


Fig. 1. Oleuropein structure.

After one week of acclimatization, rats were randomly allocated into four groups ($n = 7$) as follows: the control group received an oral administration of 0.9% NaCl for 12 consecutive days; the oleuropein group received an oral administration of oleuropein (50 mg/kg body weight.) every day for 12 days; the AKI group received an oral administration of 0.9% NaCl every day for 12 days; and the oleuropein + AKI group received an oral administration of oleuropein every day for 12 days. Rats in the control and oleuropein groups were injected intramuscularly with 0.9% NaCl on day 7, whereas rats in the AKI and oleuropein + AKI groups were injected intramuscularly with 50% glycerol (10 ml/kg body weight) after being deprived of water for 24 h. The dose of oleuropein was equivalent to 500 mg/kg of olive leaf extract. [17]. During the current experiment, no incidence of mortality, or any abnormal clinical changes were recorded following the administration of glycerol and/or oleuropein. Additionally, a decrease in the food consumption was observed in glycerol treated group. Thirteen days after the first administration, all the rats were sacrificed by cervical dislocation following sodium pentobarbital injection (Sigma-Aldrich) at a dose of 300 mg/kg of body weight. The blood was collected and the rats were then dissected to isolate the kidneys. The right kidney was homogenized for biochemical determination, while the left kidney was immediately immersed in neutral formalin for histological examination.

2.3. Relative kidney weight estimation

The relative kidney weight was determined based on the following formula:

$$\text{Relative Kidney weight} = \frac{\text{Right kidney}}{\text{Body weight}} \times 100$$

2.4. Biochemical parameters

2.4.1. Preparation of kidney homogenate

The right kidney was homogenized in 0.05 M Tris–HCl (pH 7.4) at a ratio of 1:10 (*w/v*). The homogenate was centrifuged at 4 °C for 10 min at 5000 ×*g*. The resulting supernatant was stored at −80 °C for subsequent biochemical analysis. Renal protein content was evaluated according to the method of Lowry [18]. Bovine serum albumin was used as a reference protein.

2.5. Kidney functions and RM-related markers

Serological levels of urea and creatinine, as well as the activities of lactate dehydrogenase and creatine kinase, were assayed using commercially available kits sourced from Randox Laboratories (Crumlin, UK) according to the manufacturer's protocol.

2.6. Kidney injury molecule-1 (Kim-1)

Kidney injury molecule-1 expression was determined using western blotting. Protein extraction and western blot analyses were performed as previously described [19]. The antibodies used were anti-rat TIM-1/KIM-1/HAVCR1 (AF3689, 1:500; R&D System), anti-βeta-Actin (MAB8929, 1:500; R&D System), and goat anti-mouse IgG (sc-2039, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody-antigen complex was visualized using an enhanced chemiluminescence detection kit (Bio-Rad, USA) following the manufacturer's instructions. Images were analyzed using Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA). β-Actin was used as an internal

control for total proteins, and the data were expressed as a percent of controls.

2.7. Oxidant/antioxidant status analysis

As a measure of lipid peroxidation, malondialdehyde levels were determined according to the protocol described by Ohkawa et al. [20]. The nitric oxide (NO; nitrite/nitrate) level was determined using Griess reagent based on the method described by [21]. The GSH content in the kidney tissue was estimated based on the method described in Ellman [22]. The activities of renal antioxidant enzymes, including superoxide dismutase, were determined according to the method of Nishikimi et al. [23], catalase activity was assayed by estimating the decomposition rate of hydrogen peroxide at 240 nm, according to the procedures described by Aebi [24]. Finally, the activities of glutathione peroxidase and glutathione reductase were assessed utilizing the methods of Paglia and Valentine [25] and De Vega et al. [26], respectively.

2.8. Measurement of myeloperoxidase (MPO) activity

Myeloperoxidase activity was measured as a marker of leucocyte migration and aggregation. Renal tissue was homogenized at a ratio of 1:20 (*w/v*) in ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%). The resulting homogenate was frozen and thawed three times, and then centrifuged at 5000 ×*g* for 25 min at 4 °C. Myeloperoxidase activity in the supernatant was measured using *O*-dianisidine according to the method of Bradley et al. [27]. Changes in absorbance at 460 nm were recorded for 3 min. Myeloperoxidase activity data were expressed in units per mg of protein.

2.9. Pro-inflammatory cytokines/chemokine biomarker assay

Renal quantification of chemokine (C−C motif) ligand 2 (CCL2), tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL1β), and interleukin 2 (IL2) were assessed using commercially available ELISA kits supplied by Novus Biologicals (Centennial, CO, USA) based on the manufacturer's protocols.

2.10. Estimation of apoptotic proteins

The levels of the anti-apoptotic protein (Bcl-2) and the pro-apoptotic proteins BCL2-associated X protein (Bax) and caspase 3 were estimated using a commercially available ELISA kit provided by Cusabio (Wuhan, China) according to the manufacturer's instructions.

2.11. Real-time PCR

Total renal RNA was isolated using TRIzol reagent according to the method described by Chomczynski et al. [28]. The quality and quantity of isolated RNA were determined using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA), and the RNA was immediately reverse transcribed to cDNA using RevertAid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Canada) according to the manufacturer's instructions. For gene expression analysis, quantitative real-time PCR was employed using the QuantiFast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Forward and reverse primers were obtained from Jena Bioscience (Jena, Germany) and are listed in Supplementary Table 1. All reactions were performed in triplicate using a ViiA™ 7 System (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a standard reference gene.

2.12. Histological procedures

Left kidneys were fixed in 10% neutral formalin for 24 h and then dehydrated using high-grade alcohol, embedded in paraffin, sectioned (8-μm sections), and stained with hematoxylin and eosin. Finally, the slides were examined using a Nikon Eclipse E200-LED microscope (Tokyo, Japan) microscope at ×400 magnification.

2.13. Immunohistochemistry

To evaluate the expression of caspase-3, the pro-apoptotic protein. The prepared renal sections with 5-μm thickness were blocked with hydrogen peroxide (0.1%) containing methanol for 15 min to damage the endogenous peroxidase. After blocked sectoins were incubated with a rabbit polyclonal Bax antibody at 4 °C for 12 h. Thereafter, the sections were washed with phosphate-buffered saline and incubated with biotinylated goat anti-rabbit immunoglobulins, followed by streptavidin–peroxidase complexes at 30 °C for 30 min. The peroxidase activity was developed using diaminobenzidine (DAB)-hydrogen peroxide. Images were recorded at an original magnification of 400× using a Nikon microscope (Eclipse E200-LED, Tokyo, Japan).

Immunohistochemistry was investigated on dewaxed glass slides. The antigen sites were revealed by washing sections with boiled water followed by 0.03% H_2O_2 in absolute methanol for 10 min. Sections were kept at 4 °C overnight with (1:50) polyclonal rabbit anti-caspases-3 antibody (Santa Cruz, CA, USA). To remove the unbound primary antibodies, sections were washed with phosphate buffer saline (PBS), then incubated for 30 min with goat-derived secondary anti-rabbit antibody conjugated to horseradish peroxidase at 37 °C. The interactions between antigen and antibody were recognized by incubating sections for 10 min at room temperature with the chromogen 3,3′-diaminobenzidine tetrachloride (DAB-H_2O_2) as substrate. With the Nikon Eclipse E200-LED, the renal sections were examined using 400 x magnification lens. In the current investigation, the renal sections were incubated under the same conditions and at the same time with the same antibodies concentration to guarantee that the immunostaining would be comparable between the different treated groups.

2.14. Statistical analysis

The data are presented as means ± standard deviation (SD) of seven rats. Differences between the different groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range *post-hoc* test. The Student's *t*-test was used to compare differences between means. A significant difference was considered when $P < 0.05$.

3. Results

3.1. Oleuropein inhibited the changes in relative kidney weight in response to glycerol injection-induced AKI

In the current study, a single dose of 50% glycerol, injected intramuscularly (10 ml/kg of body weight), was used to induce RM, and subsequently AKI, in rats. A significant increase ($P < 0.05$) in the relative weight of kidneys was observed compared to that in normal rats following glycerol administration. Meanwhile, the relative kidney weight of rats administered oleuropein for 12 days at a dose of 50 mg/kg of body weight showed no change. However, a significant decrease in kidney relative weight was recorded in the oleuropein + glycerol-treated group compared to that in glycerol-only intoxicated rats (Fig. 2).

3.2. Effect of oleuropein on RM- and AKI-related markers following glycerol injection

To confirm the induction of RM, and subsequently AKI, following glycerol injection, the levels of creatine kinase, lactate dehydrogenase, urea and creatinine were estimated. Additionally, Kim-1 which is a transmembrane protein and widely used as a specific indicator of renal

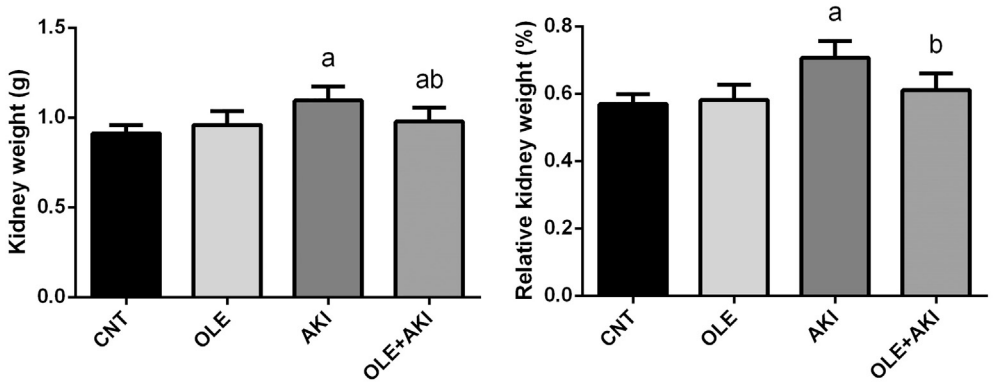
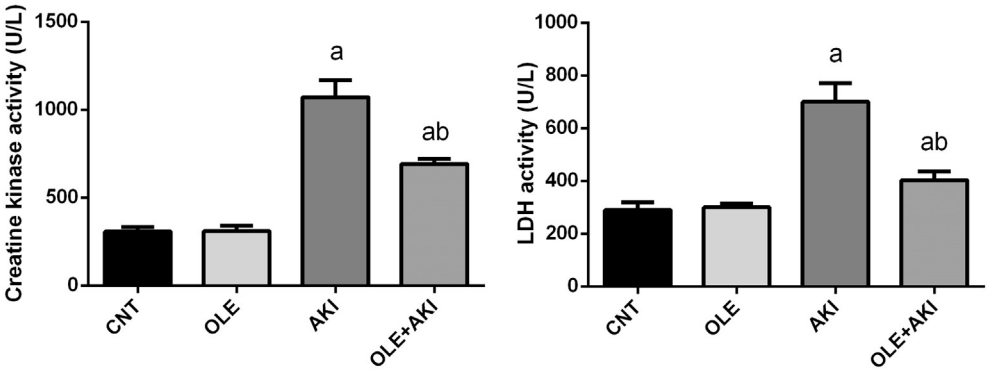


Fig. 2. Effects of oleuropein (50 mg/kg) on kidney relative weight in a glycerol-induced acute kidney injury rat model. Data are presented as the means ± SD ($n = 7$); $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group.



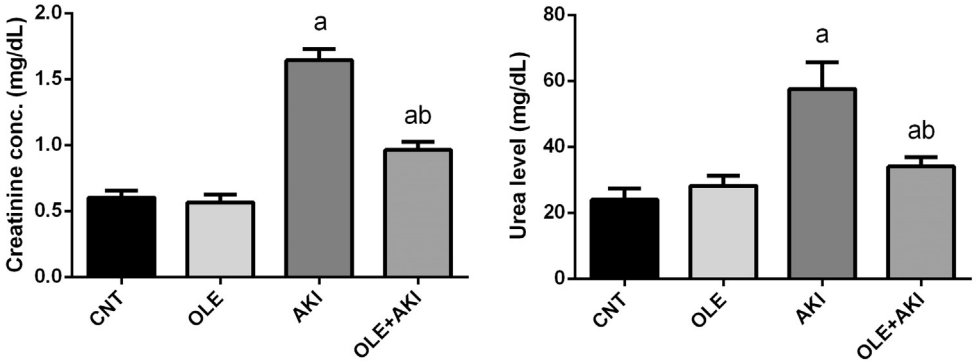


Fig. 3. Effects of oleuropein (50 mg/kg) on the levels of rhabdomyolysis and acute kidney injury-related parameters, including creatine kinase, lactate dehydrogenase, creatinine, and urea. Data are presented as the means ± SD (n = 7); $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group.

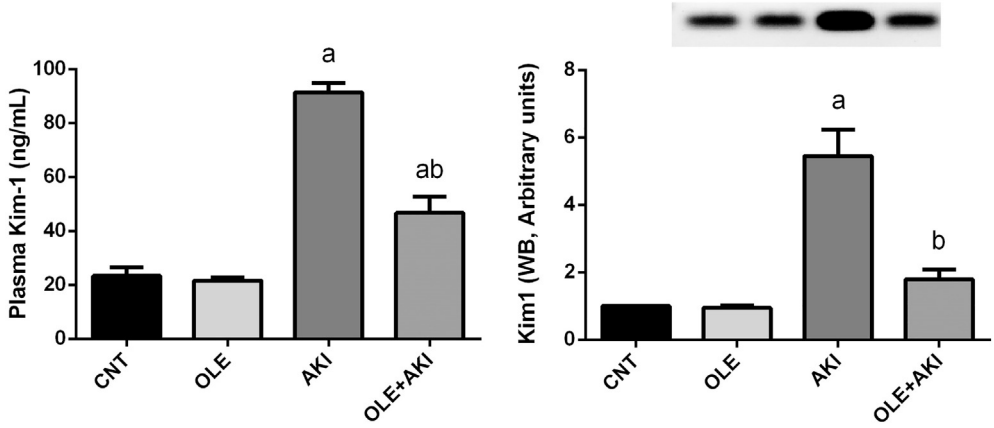


Fig. 4. Kim-1 level and expression were determined using ELISA and western blot. Data are presented as the means ± SD; $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group.

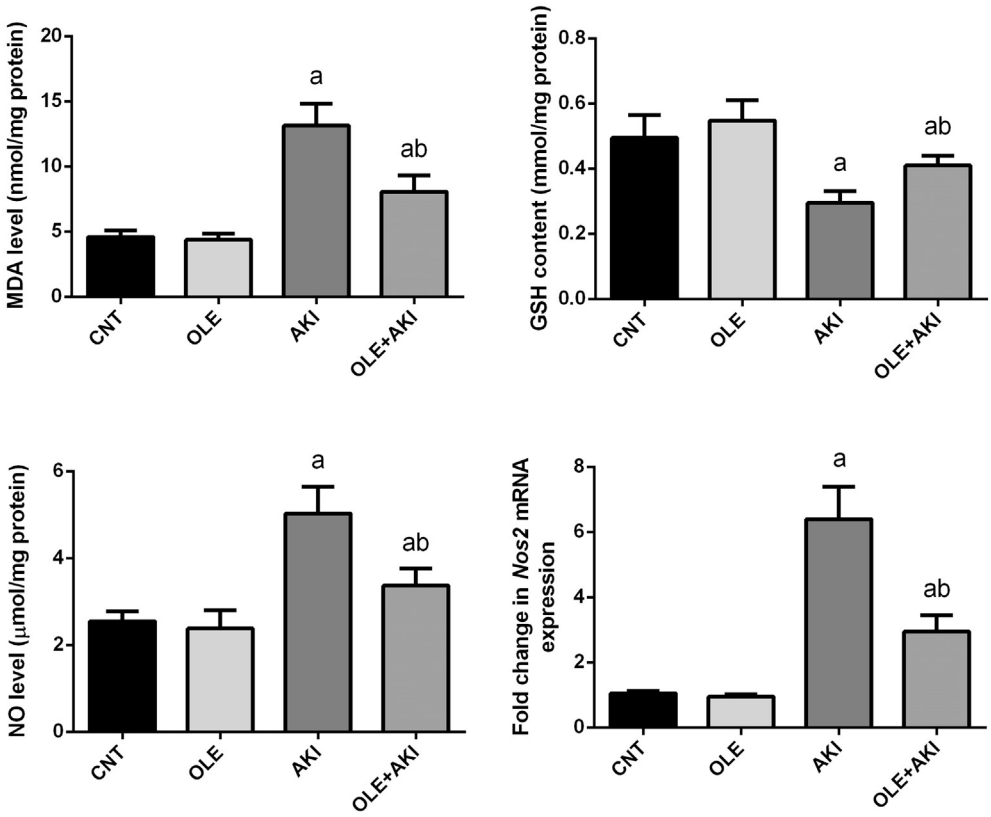


Fig. 5. Effects of oleuropein (50 mg/kg) on the levels of malondialdehyde, NO, and GSH in renal tissue following glycerol injection. Data are presented as the means ± SD (n = 7); $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group.

injury was estimated. Glycerol-injected rats showed a marked and significant increase ($P < 0.05$) in RM-related indices, including levels of creatine kinase and lactate dehydrogenase. Moreover, kidney function markers, including levels of urea, creatinine, and Kim-1 expression were significantly increased compared to the control group. Administration of oleuropein resulted in significantly reduced alterations in the levels of RM- and AKI-related parameters compared to the glycerol-only treated group, reflecting its ability to protect muscular and renal tissues in response to glycerol intoxication (Figs. 3 and 4).

3.3. Effect of oleuropein on the oxidative status following glycerol injection

The results shown in Figs. 5 and 6 confirmed the involvement of oxidative stress as a fundamental mechanism in AKI pathophysiology. Glycerol-intoxicated rats showed a significant increase in malondialdehyde and NO levels compared to the control levels, whereas a significant decrease was observed in GSH levels. Moreover, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities and their gene expression were downregulated in renal tissue following glycerol injection. Notably, oral administration of oleuropein prior to glycerol injection restored the imbalance between oxidant and antioxidant activity, as evidenced by reduced malondialdehyde and NO levels, as well as increased GSH content and superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities and their gene expression, compared to the glycerol-intoxicated group.

To further investigate the oleuropein-mediated molecular antioxidant mechanism, nuclear factor, erythroid derived 2, like 2 (*Nfe2l2)* and heme oxygenase 1 (*Hmox1*) mRNA expression was investigated using qRT-PCR. NFE2L2 protects cells from reactive oxygen species (ROS) through different pathways, while HMOX1 plays an important role in heme metabolism. Glycerol-injected rats showed a marked downregulation in *Nfe2l2* and *Hmox1* mRNA expression levels compared to the control group; in contrast, oleuropein administration significantly upregulated the expression of these two genes in renal tissue compared to the glycerol-induced AKI group, reflecting its nephroprotective effect against oxidative reactions elicited by glycerol injection in the renal tissue (Fig. 7).

3.4. Effect of oleuropein on inflammatory responses following glycerol injection

An inflammatory response was recorded in the renal tissue following intramuscular glycerol injection. Fig. 6 illustrates that rats exposed to glycerol exhibited a significant increase in the transcription levels of pro-inflammatory mediators, including *Tnf*, *Il1B, Il2,* and nitric oxide synthase 2, inducible (*Nos2*), in renal tissue compared to those in the control group. In addition, the ELISA assay showed that glycerol injection elicited increases in the levels of TNF, IL1B, and IL2. Furthermore, glycerol administration resulted in elevated CCL2 content and MPO activity. Interestingly, oleuropein injection led to a significant inhibition of the glycerol-induced inflammatory reactions, indicating it possesses potent anti-inflammatory activity and could therefore be used as an alternative therapeutic agent to counteract the inflammation associated with AKI development (Fig. 8).

3.5. Effect of oleuropein on apoptotic signaling in response to glycerol injection

To evaluate the apoptotic events following glycerol intoxication, the transcriptional levels of pro-apoptotic (Bax and caspases-3) and the anti-apoptotic B cell leukemia/lymphoma 2 (Bcl-2) proteins were assessed in the kidney tissue. Our ELISA and qRT-PCR results revealed that glycerol intoxication elicited a significant increase in the protein and mRNA expression levels of Bax and caspases-3, whereas those of Bcl-2 were downregulated, compared to the control group. Caspase-3 expression was also evaluated using the immunohistochemistry to

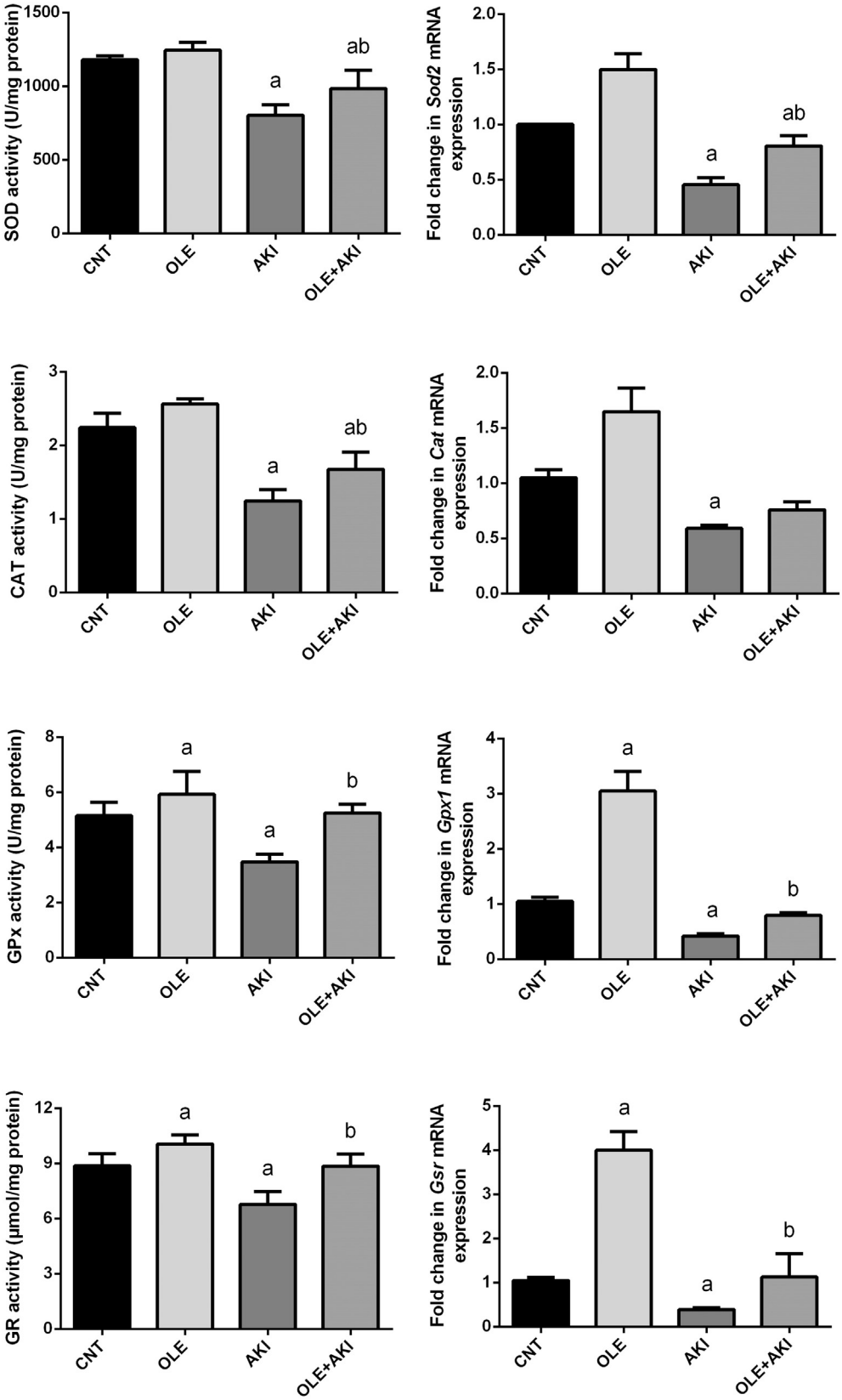


Fig. 6. Effects of oleuropein (50 mg/kg) on GPx, GR, SOD, and CAT activities and their gene expression in renal tissue following glycerol injection. $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group. mRNA expression results are presented as the means ± SD of three assays referenced to *Gapdh* and represented as fold changes (log2 scale) compared to the mRNA levels of the control.

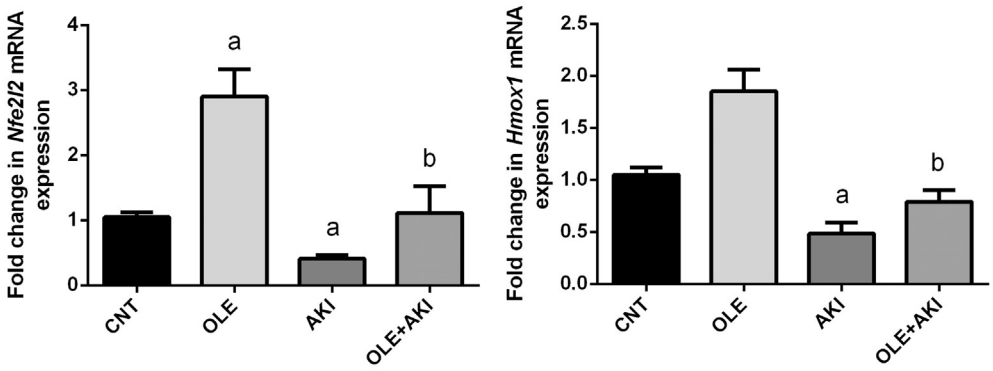


Fig. 7. Effects of oleuropein (50 mg/kg) on *Nfe2l2* and *Hmox1* mRNA expression in renal tissue following glycerol injection. [a]$P < 0.05$, compared to the control group; [b]$P < 0.05$, compared to the glycerol-injected group. mRNA expression results are presented as the means ± SD of three assays referenced to *Gapdh* and represented as fold changes (log2 scale) compared to the mRNA levels of the control.

further validate our results. Glycerol application was found to increase caspases-3 immunoreactivity compared to the control group. Interestingly, when oleuropein was administered before glycerol, apoptotic reactions were significantly inhibited in the kidney tissue, as evidenced by the decreases in Bax and caspases-3 and increases in Bcl-2 compared to the glycerol-induced AKI group (Figs. 9 and 10).

3.6. Effect of oleuropein on the histological changes occurring in response to glycerol injection

The histopathological microscopic results of the kidney tissue in the experimental groups and in control rats are shown in Fig. 10. Kidney tissue sections obtained from control and oleuropein-treated rats showed normal renal tissue architecture, characterized by an intact renal tubular epithelium and no evident pathological alteration in the glomerular or renal interstitium (Fig. 11a and b). In contrast, kidney sections from glycerol-injected rats showed widespread damage, evidenced by tubular dilatation and vacuolation, glomerular hypertrophy, debris in the renal tubular lumen, and severe interstitial inflammatory infiltration (Fig. 11c). Oleuropein pretreatment markedly inhibited the severity of renal injuries (Fig. 11d).

4. Discussion

Rhabdomyolysis is characterized by extensive skeletal muscle fiber destruction and the release into the blood of its contents, including lactate dehydrogenase, myoglobin, glutamic oxalacetic transaminase, creatine kinase, glutamic oxalacetic transaminase, aldolase, and electrolytes. These contents are filtered out through glomeruli and enhance the development of AKI, which is associated with a high rate of mortality worldwide [5]. Therefore, adequate and safe drugs to treat or minimize AKI and its causes must be found. This study was conducted to evaluate the potential renoprotective effect of oleuropein against RM-induced AKI in rats following glycerol administration. The results showed a marked increase in kidney relative weight and markers of renal function, including serum creatinine, urea, and Kim expression. This was accompanied by increases in serological RM-related biomarkers, including lactate dehydrogenase and creatine kinase activities, in response to glycerol injection. Glycerol is a nephrotoxic agent that disrupts glomerular filtration, triggers renal tubule damage, and elicits swelling of stromal and epithelial cells, thus increasing kidney weight [29]. The increased serum creatinine and blood urea nitrogen levels represent important renal injury markers [8,30]. The overexpression of Kim is used as a specific indicator of renal injury [31]. An increased kidney function index has been attributed to impairment of the tubular membranes, which was also observed in this study. Disruption of plasma and subcellular membranes is suggested to be strongly linked to AKI pathophysiology [32]. Increased creatine kinase and lactate dehydrogenase content result from damaged skeletal muscle fibers that may occur due to muscle trauma, disruption of muscle fiber metabolism, or the effect of toxicants. These products induce oxidative reactions, inflammation responses, activation of pro-apoptotic proteins, vasoconstriction, and tubular obstruction, all of which further aggravate AKI [33].

Although animal models have suggested several strategies for the treatment of glycerol-induced renal injury, mainly through antioxidant agents, clinical application of these strategies is still limited [34]. Decreasing kidney function parameters are indicative of improved renal function following toxicant exposure. In this context, oleuropein administration was found to reverse an increase in body weight and creatinine and urea levels following cisplatin- and bisphenol A-induced renal injury [35,36].

The kidney is highly susceptible to oxidative injury generated by ROS due to its high polyunsaturated fatty acid content [37]. Renal damage can also result from high renal blood flow and filtration of large quantities of xenobiotics that can accumulate in renal lobules [38]. In the current investigation, intramuscular injection of glycerol enhanced oxidative stress. In fact, glycerol resulted in myoglobin release into the circulation in large amounts which is converted inside the tubular cells to its oxidized form and enhances ROS production, triggering membrane lipid peroxidation and additional malondialdehyde formation [5,39]. Myoglobin-induced lipid peroxidation has been linked to F2-isoprostane production, which has a vasoconstrictive effect characteristic of AKI [33]. ROS are also known to enhance the expression of inducible nitric oxide synthase, the rate-limiting enzyme in NO synthesis. The oversecreted NO interacts with superoxide to form peroxynitrite radicals, resulting in further renal tubular damage in glycerol-induced AKI [40,41]. Moreover, the increased oxidative reactions may lead to the depletion and deactivation of cellular defense mechanisms [42]. Decreased enzymatic and non-enzymatic molecules activities in kidney tissue following glycerol injection have been reported previously [43,44]. The authors attributed the reduced response of the cellular antioxidant defense system to ROS overproduction, increased lipid peroxidation, and downregulation of associated gene expression, which was also shown in this study.

Our results showed a decrease in *Nfe2l2* and *Hmox1* mRNA expression in renal tissue following glycerol administration. Nuclear factor erythroid 2–related factor 2 (Nrf2) protects cells from ROS through different pathways, including GSH synthesis, upregulation of antioxidant and detoxifying molecules, and degradation of superoxide and peroxide radicals by GPx and SOD [45]. Heme oxygenase 1 (HO-1) is a rate-limiting enzyme that converts heme into carbon monoxide, iron, and biliverdin, which is further converted to bilirubin by biliverdin reductase. Carbon monoxide acts as a vasodilator and suppresses platelet aggregation [46]. Furthermore, HO-1 has a cytoprotective effect in renal injury *via* its antioxidant and anti-inflammatory effects [47]. However, our results showed that oleuropein restored the oxidant/antioxidant balance in kidney tissue. Our results suggest that antioxidant-based therapy may prevent lipid peroxidation-mediated oxidative challenge and the subsequent worsening of complications

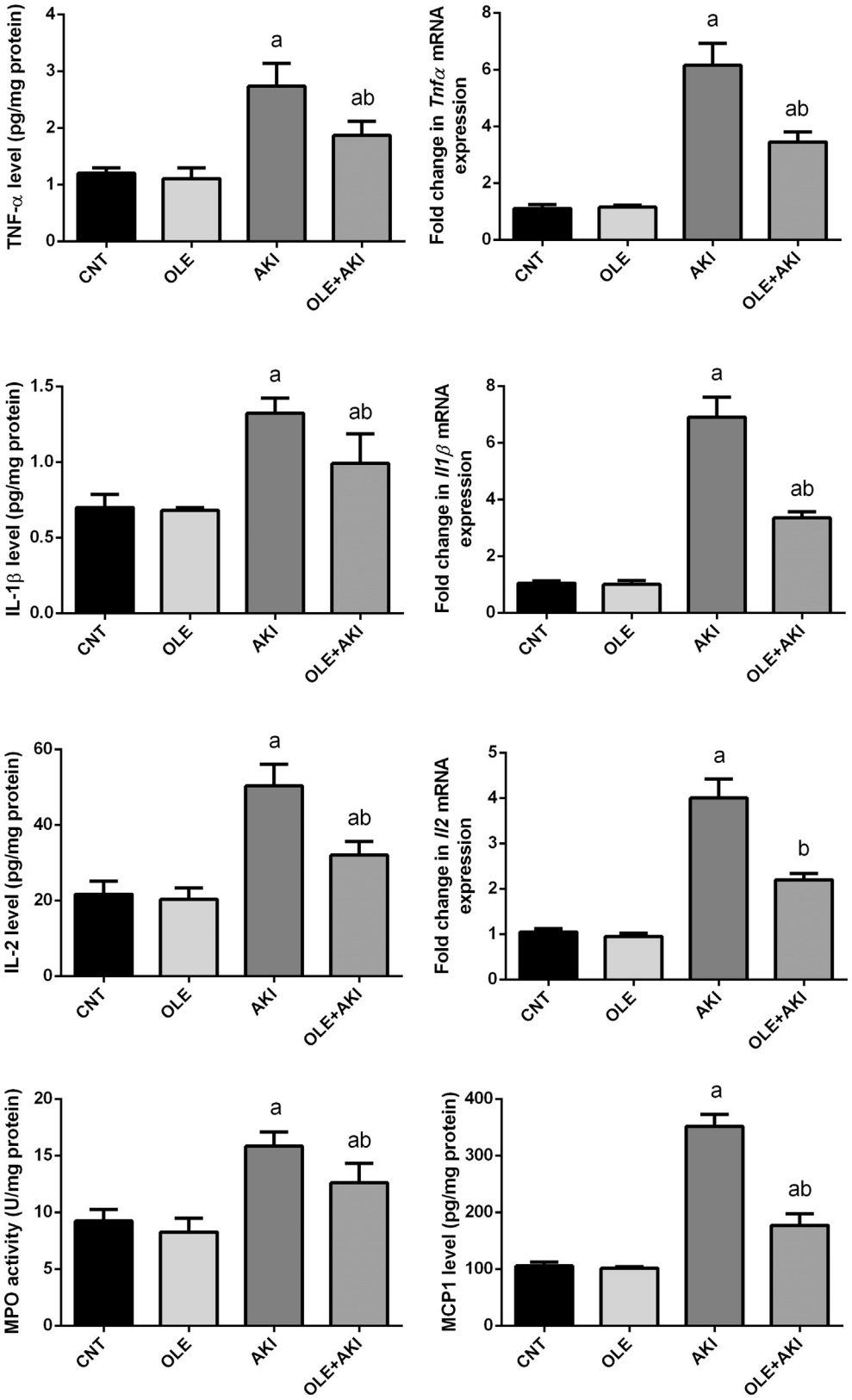


Fig. 8. Effects of oleuropein (50 mg/kg) on inflammatory mediators in renal tissue, including the protein and mRNA expression levels of *Tnf*, *Il1b*, *Il2*, *Ccl2*, and MPO activity and following glycerol injection. $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group. mRNA expression results are presented as the means ± SD of three assays referenced to *Gapdh* and represented as fold changes (log2 scale) compared to the mRNA levels of the control.

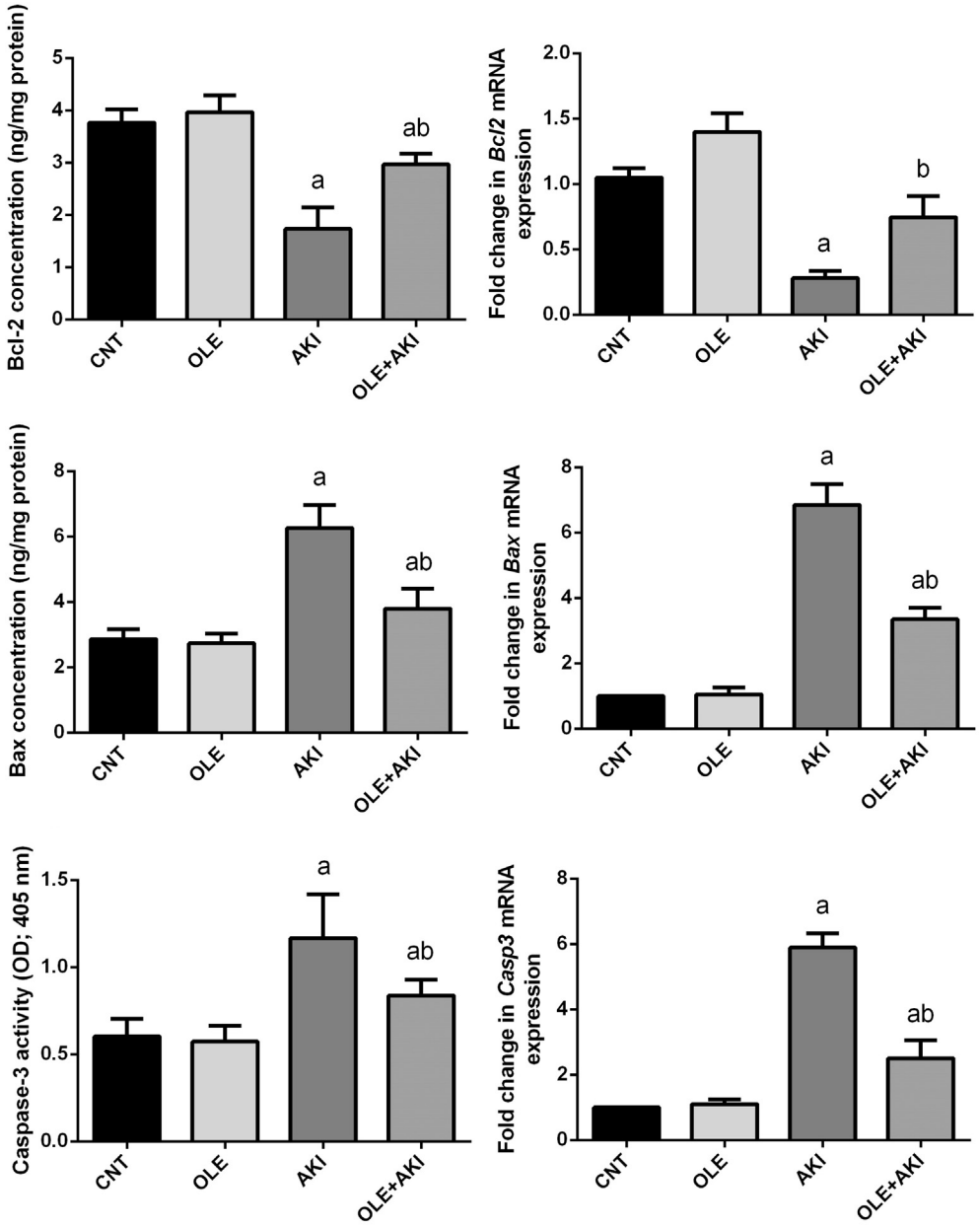


Fig. 9. Effects of oleuropein (50 mg/kg) on the levels and expression of apoptotic proteins in renal tissue following glycerol injection. $^{a}P < 0.05$, compared to the control group; $^{b}P < 0.05$, compared to the glycerol-injected group. mRNA expression results are presented as the means ± SD of three assays referenced to *Gapdh* and represented as fold changes (log2 scale) compared to the mRNA levels of the control.

associated with RM-induced AKI [42]. The hydroxytyrosol moiety in oleuropein is responsible for its antioxidant activity through forming intramolecular hydrogen bonds with the free radicals [48]. Meanwhile, the pharmacological activity has been attributed to the presence of the ortho-diphenolic system (catechol) [49]. Oleuropein enhances the antioxidant response through scavenging of free radicals, inhibition of lipid peroxidation, and increases in the levels of antioxidant defense molecules and their gene expression in different experimental conditions [50,51]. Moreover, oleuropein activates NFE2L2 signaling, thus inhibiting oxidative stress progression and inflammatory responses in rats with cyclophosphamide-induced hepatic injury [52].

Cellular damage and associated molecules are suggested as key inflammation stimulators following acute tissue injury [53]. In the present study, an inflammatory response was recorded in kidney tissue following glycerol injection. Increased levels of *Ccl2*, a chemotactic factor, have been linked to renal inflammatory disease through directing monocytes from the bone marrow to the site of inflammation [54]. MPO is a heme-containing peroxidase used widely as an indicator of oxidative stress and inflammation [55]. The MPO level was elevated in inflamed renal tissue, with monocytes representing the main source of MPO [34,56]. In RM, increased concentrations of TNF, IL1B, and IL2 have been attributed to several factors, including myoglobin-derived heme following myocyte destruction, ROS production, and activation of nuclear factor-kappa B (NF-κB), that trigger overexpression of *Tnf*, *Il1b*, and *Il2,* leading to renal injury [44,57,58]. Oleuropein administration decreased the inflammatory responses in renal tissue following glycerol intoxication. The anti-inflammatory activity was also exerted *via* decreasing MPO activity and downregulating *Ccl2* expression. Numerous studies have reported that oleuropein exerts its anti-inflammatory activity by suppressing the production and activities of several inflammatory mediators, including NF-κB, TNF, IL1B, and NOS2 [59–61]. *Ccl2* inhibition is associated with renal injury amelioration in several

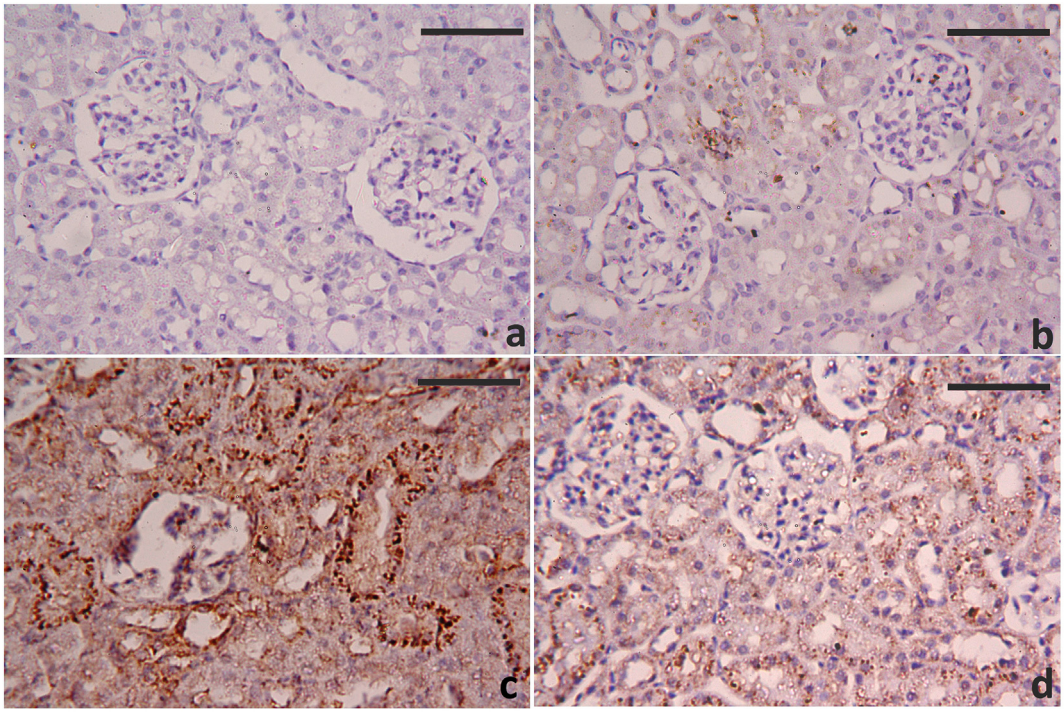


Fig. 10. Expression of caspase-3 in oleuropein- and/or glycerol-treated groups using immunohistochemistry technique. Kidney tissue sections obtained from (a) control and (b) oleuropein-treated rats showed normal caspase-3 expression. Meanwhile, kidney sections from (c) glycerol-injected rats showed overexpression of caspase-3. Interestingly, (d) oleuropein pretreatment significantly downregulated the expression of caspase-3 in the renal tissue response to glycerol injection.

models, suggesting that this mechanism could be used to treat or minimize renal inflammatory diseases, including AKI [54]. Oleuropein decreased MPO activity, leukocyte infiltration, and glomerulosclerosis in renal tissue of diabetic rats [62].

Apoptosis has been implicated as a key factor driving RM development, and thereby AKI. Intramuscular injection of glycerol triggered apoptotic reactions in the kidney tissue. Lipid peroxidation in RM is reported to disrupt mitochondrial membrane permeability, resulting in

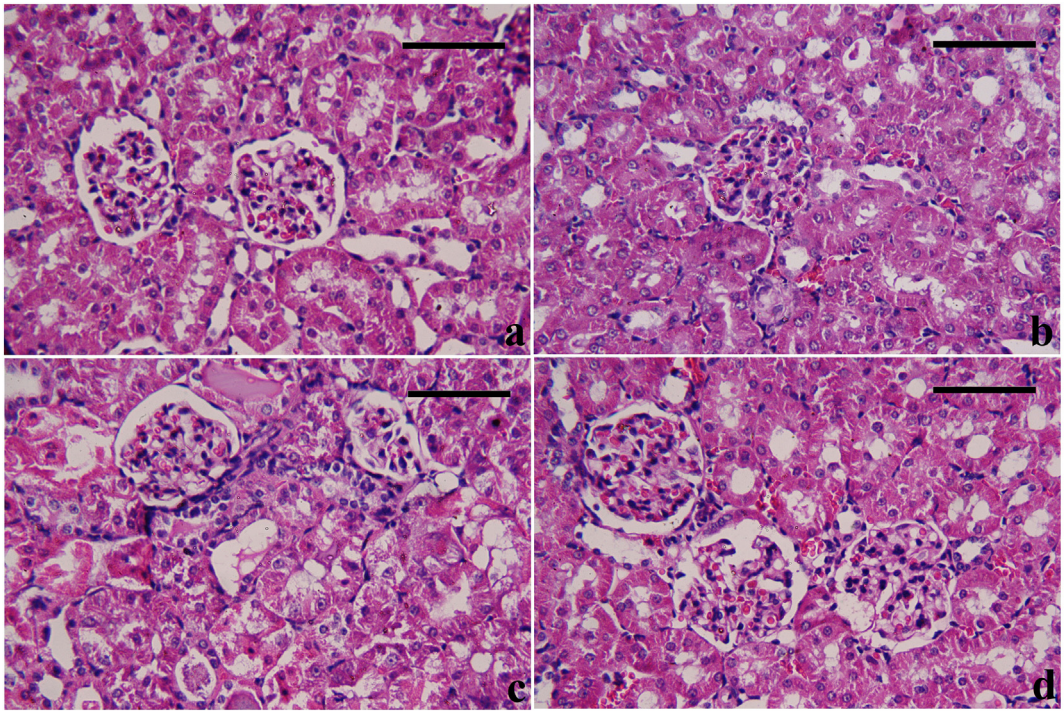


Fig. 11. Histopathological changes in oleuropein- and/or glycerol-treated groups. Sections were stained with hematoxylin and eosin (×400). Kidney tissue sections obtained from (a) control and (b) oleuropein-treated rats showed normal renal tissue architecture, characterized by an intact renal tubular epithelium and no evident pathological alteration in the glomerular or renal interstitium. In contrast, kidney sections from (c) glycerol-injected rats showed widespread damage, evidenced by tubular dilatation and vacuolation, glomerular hypertrophy, debris in the renal tubular lumen, and severe interstitial inflammatory infiltration. (d) oleuropein pretreatment markedly inhibited the severity of renal injuries.

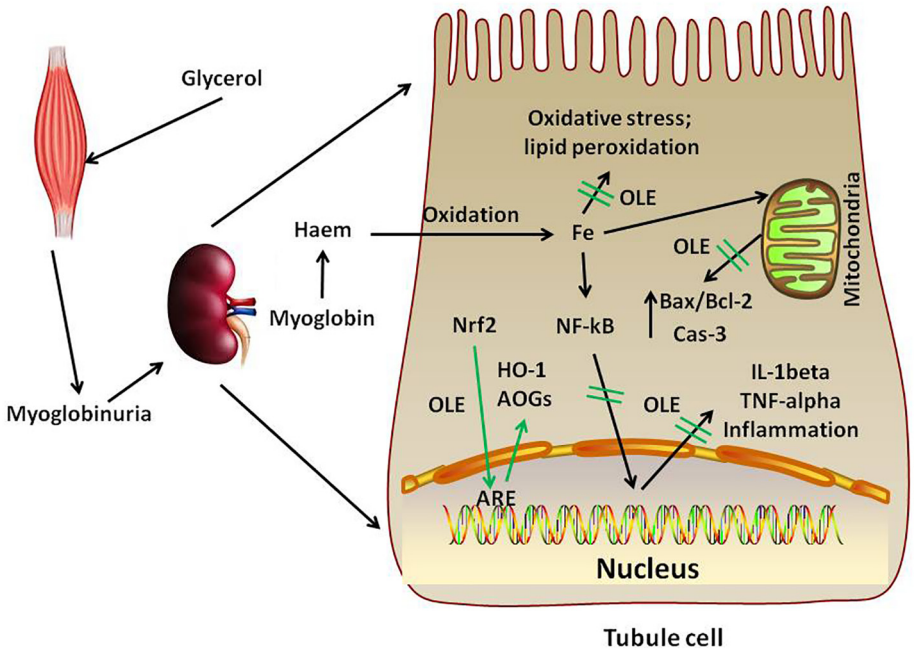


Fig. 12. A summarized illustration describes the possible protective effect of oleuropein as an integrated multi-functional renoprotective agent in AKI model in rats. OLE: oleuropein; Nrf2: nuclear factor erythroid 2–related factor 2; HO-1: homoxgenase-1; AOGs: antioxidants genes; ARE: antioxidant response elements; NF-κB: nuclear factor kappa B; 1L-1beta: interleukin-1 beta; TNF-alpha: tumor necrosis factor-alpha; Bax: BCL2-associated X protein; Bcl-2: *B-cell lymphoma 2; Cas-3: caspase-3.*

ROS overproduction, cytochrome *c* release, activation of pro-apoptotic proteins, and enhanced tubular cell apoptosis [63]. Because ROS production enhances apoptotic reactions in renal tissue, using antioxidant molecules has been suggested as a means of minimizing or preventing AKI [64]. Oleuropein reversed the alterations in the expression of apoptotic proteins following glycerol intoxication. Oleuropein reportedly suppresses the apoptotic cascade in response to cisplatin-triggered renal damage by inhibiting Bax and caspases-3 expression [60]. The anti-apoptotic effect of oleuropein has been attributed to its capacity to quench ROS that trigger mitochondrial apoptotic reactions [65]. Consequently, based on the above results, Fig. 12 schematically illustrates the possible protective effect of OLE as an integrated multi-functional renoprotective agent.

5. Conclusion

A variety of mechanisms have been implicated in the pathophysiology of RM-induced AKI. Our findings revealed changes in RM-related markers, including creatine kinase and lactate dehydrogenase activities, and increases in kidney function indices, including increases in the levels of creatinine, urea, and *Havcr1* expression in response to glycerol injection. An imbalance between oxidant and antioxidant levels was also observed (increased malondialdehyde and NO, and decreased GSH, SOD, CAT, GPx, and GR activities and their gene expression). Inflammation-related signaling was recorded in the kidney tissue (increases in the protein and mRNA expression levels of TNF-α, IL1β, and IL2, as well as MPO activity and *Ccl2* expression), as were apoptotic events (upregulation of Bax and caspases-3 and downregulation of Bcl-2 expression). In contrast, oleuropein pre-administration was able to alleviate these alterations in the kidney tissue through its potent antioxidant capacity, anti-inflammatory effects, and anti-apoptotic activity. Taken together, our data demonstrated that oleuropein merits further consideration as a potential therapeutic agent to prevent or minimize the progression of AKI.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116634.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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