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Usnic acid alleviates testicular ischemia/reperfusion injury in rats by modulating endoplasmic reticulum stress

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ABSTRACT

Testicular torsion (TT) is a urological condition that can result in infertility in men. The etiopathogenesis of TT includes ischemia/reperfusion injury (IRI) characterized by oxidative stress (OS), inflammation and apoptosis resulting from increased levels of free radicals. Usnic acid (UA), a dibenzofuran, is one of the most common metabolites found in lichens and is known to possess powerful antioxidant properties. The aim of this study was to investigate the potential protective activity of UA in an experimental testicular IRI model for the first time. A total of 18 rats were randomly assigned to three groups (n=6): sham control, IRI and IRI+UA. The IRI groups underwent a four-hour period of ischemia and a two-hour period of reperfusion. The OS, inflammation, endoplasmic reticulum stress (ERS) and apoptosis markers in testicular tissue were evaluated using colorimetric methods. Furthermore, tissue samples were subjected to histological examination, with staining using hematoxylin and eosin. Histopathological findings supported by increased OS, inflammation, ERS and apoptosis levels were obtained in IRI group compared with sham control group. However, UA treatment restored these pathological and biochemical changes. Although this study provides the first preliminary evidence that UA may be used as a useful molecule against testicular IRI, further extensive molecular preclinical studies should be performed before clinical use is considered.

Abbreviations: ATF6, activating transcription factor 6; CAT, catalase; DDIT3, DNA damage-inducible transcript 3; DMSO, dimethyl sulfoxide; ERS, endoplasmic reticulum stress; GPx, glutathione peroxidase; GSH, glutathione; H&E, hematoxylin and eosin; HSPA5, heat shock protein family A HSP70 member 5; IL-6, interleukin-6; IRI, ischemia/reperfusion injury; MDA, malondialdehyde; MPO, myeloperoxidase; OS, oxidative stress; OSI, oxidative stress index; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; T/D, torsion/detorsion; TNF-α, tumor necrosis factor-alpha; TOC, total oxidant capacity; TT, testicular torsion; UA, usnic acid; UPR, unfolded protein response.

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1. Introduction

Testicular torsion (TT) is a urological emergency that occurs when the spermatic cord bends along its axis, with a higher incidence observed in newborns and adolescent males [1]. TT is estimated to affect 1 in 4000 men under the age of 25 [2]. Given that rotation of the testicle and twisting of the spermatic cord can cause testicular ischemia and death of germ cells, it is imperative that a rapid diagnosis is made and urgent surgical intervention is carried out [3]. Following TT, the testicular salvage rate is 90–100 % if intervention occurs within the first 4–6 hours. However, this rate can decrease to 20–50 % in the 6–12 h period and to <10 % in the 12–24 h period [4,5]. A reduction in testicular blood flow resulting from torsion leads to a depletion of the ATP pool and an increase in reactive oxygen species (ROS), which in turn causes ischemic testicular damage and loss [6]. Although detorsion is the only mandatory option in emergency surgical intervention, testicular reperfusion provided by detorsion paradoxically causes ischemia/reperfusion injury (IRI), which further increases tissue damage [6,7]. The mechanisms underlying IRI are complex, and the main current recommendations include Ca^{2+} overload, overproduction of ROS, increased inflammation and apoptosis [1,3,6]. Therefore, testicular IRI can lead to atrophy, infertility and androgen deficiency [8]. The endoplasmic reticulum (ER) is a vital organelle that interacts with other organelles, such as the Golgi and mitochondria, and responsible for synthesis, folding, modification and targeting of proteins [9]. The IRI is a potent stressor that impairs ER homeostasis by negatively affecting the folding processes of proteins in the ER, which is highly sensitive to stress, and causes ER stress (ERS) [10,11]. The cells activate the unfolded protein response (UPR) pathway, an adaptive pathway to cope with increased ERS. The UPR aims to eliminate ERS by increasing chaperone synthesis in the acute phase and accelerating the proteasomal degradation of misfolded proteins [9]. In the event of prolonged and irremediable ERS, UPR initiates caspase-dependent apoptotic pathways through the induction of increased expression of DNA damage-inducible transcript 3 (DDIT3, also called as CHOP) [11]. Recent advances reveal that ERS is an important signaling pathway in the occurrence of IRI-induced cell damage [10–12]. Therefore, the identification of antioxidant molecules that target UPR components and have the potential to reduce ERS represents an innovative strategic approach in the treatment of IRI-associated pathologies [13–15].

Lichens are compound organisms formed from the symbiotic relationship of a fungus (mycobiont) with a photosynthetic partner (phytobiont), usually green algae or cyanobacteria [16]. Lichens produce numerous bioactive substances, such as amino acid, lactone, quinone, dibenzofuran, chromone, terpenoid, steroid and carotenoid [17]. Usnic acid (UA), a dibenzofuran derivative, is one of the most abundant characteristic secondary metabolites in lichens and widely used in the cosmetic industry [18]. It is well established that chronic use of high doses of UA can result in liver toxicity. It is therefore recommended that acute use of low doses of UA be employed [16,18]. The studies have demonstrated that UA has a number of important functions, including antimicrobial, antioxidant, anti-inflammatory and anticancer activities [17,19]. Recent studies have demonstrated the therapeutic efficacy of UA in a range of experimental models of IRI [20–22]. Nevertheless, the impact of UA on testicular IRI has yet to be demonstrated. The objective of this study was to ascertain whether UA exerts a protective effect against testicular IRI and to elucidate the underlying mechanisms, should UA indeed possess testicular protective activity.

2. Materials and methods

2.1. Animals

All experimental procedures were conducted in accordance with the ARRIVE guidelines and were approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (Protocol Number: 2022/43). A total of 18 adult male Sprague-Dawley rats (aged 8–10 weeks, 180–200 g) were housed under standard laboratory conditions, which included a 60 % relative humidity, a temperature of 22±2°C, and a 12-h light-dark cycle. All animals were subjected to a one-week acclimation phase prior to the commencement of the study. During this period, rats were provided with *ad libitum* access to water and pellet rodent chow.

2.2. Experimental groups and surgical procedures

The rats were randomly allocated to three groups: sham control, IRI and IRI+UA. Each group comprised an equal number of subjects (n=6). All animals were anaesthetised using a combination of xylazine (10 mg/kg) and ketamine (50 mg/kg), and the entire surgical procedure was performed under general anaesthesia.

2.2.1. Sham control group

The rats were subjected to a surgical stress procedure. The only incision made was that of the scrotum, and the skin was immediately sutured without the application of a torsion/detorsion (T/D) procedure. At the 210th min, 10 % dimethyl sulfoxide (DMSO) was administered intraperitoneally.

2.2.2. IRI group

The T/D procedure was applied to the rats in accordance with the model proposed by Turner et al. [23]. Ischemia was created by rotating the left testicle of each rat 720° clockwise and maintained for 240 min. At the 210th minute, 10 % DMSO was administered intraperitoneally. At the conclusion of the ischemic period, the left testicle was detorsioned and returned to its normal state, and it was maintained for 120 min.

2.2.3. IRI+UA group

The same procedure was followed as in the IRI group. In contrast to the IRI group, at the 210th min, the UA (100 mg/kg) was administered intraperitoneally.

The durations of ischemia (240 min) and reperfusion (120 min) were determined according to previous reports [24–26]. UA (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving in 10 % DMSO [22]. The dose of UA used in this study was determined according to previous experimental reports which indicated that UA exhibited antioxidant and anti-inflammatory activity [27–29]. At the 360th min, the animals were euthanized via cervical dislocation. The harvested tissues were subsequently divided into two distinct portions. One portion of the homogeneous tissue sample was immersed in Bouin's solution for histological analysis, while the second portion was immediately stored at −80°C for use in biochemical analyses.

2.3. Histological analysis

Testicular samples were fixed in Bouin's solution for a period of three days. Subsequently, the samples were dehydrated and embedded in paraffin. The 5-μm sections were obtained from the paraffin-embedded samples using an automated microtome, stained with hematoxylin and eosin (H&E), and examined under a light microscope (Olympus BX51 Tokyo, Japan) [24,30]. The prepared slides were evaluated by an expert pathologist in a blinded manner for the presence of seminiferous tubular disorganization, interstitial edema, bleeding, inflammatory and degenerative processes [26,31].

2.4. Biochemical parameters

The tissues were homogenised in 3 mL of phosphate-buffered saline using a homogeniser. The supernatants were obtained by centrifuging the homogenates at 1800 x g at 4°C for 15 minutes. The protein concentration of the supernatants was determined using the bicinchoninic acid method [32]. The determination of tissue malondialdehyde (MDA)

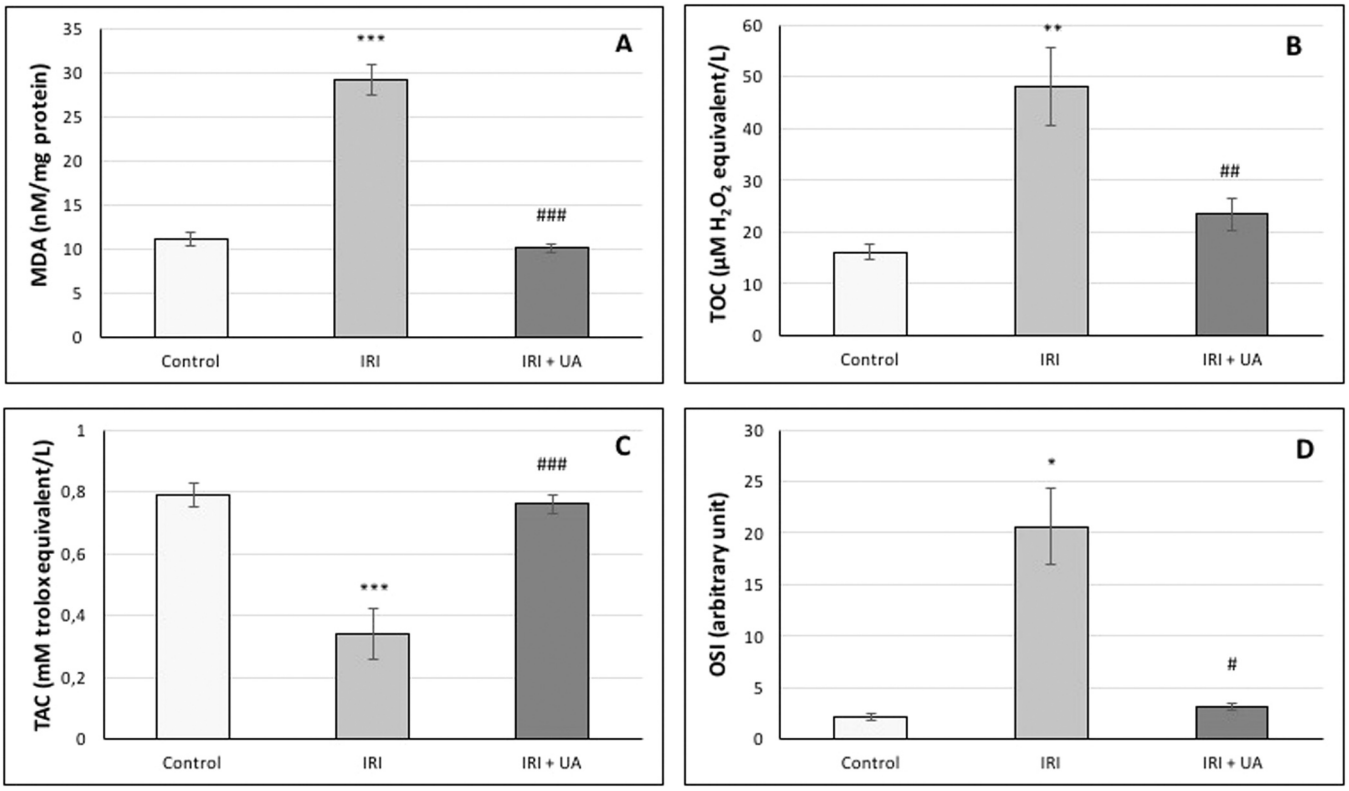

Fig. 1. Effects of treatment with UA on the OS parameters in testicular IRI (A) MDA, (B) TOC, (C) TAC, (D) OSI. All values are expressed as mean±SEM. Statistically significant from sham control group *p<0.05, **p<0.01 and ***p<0.001. Statistically significant from IRI group #p<0.05, ##p<0.01 and ###p<0.001.

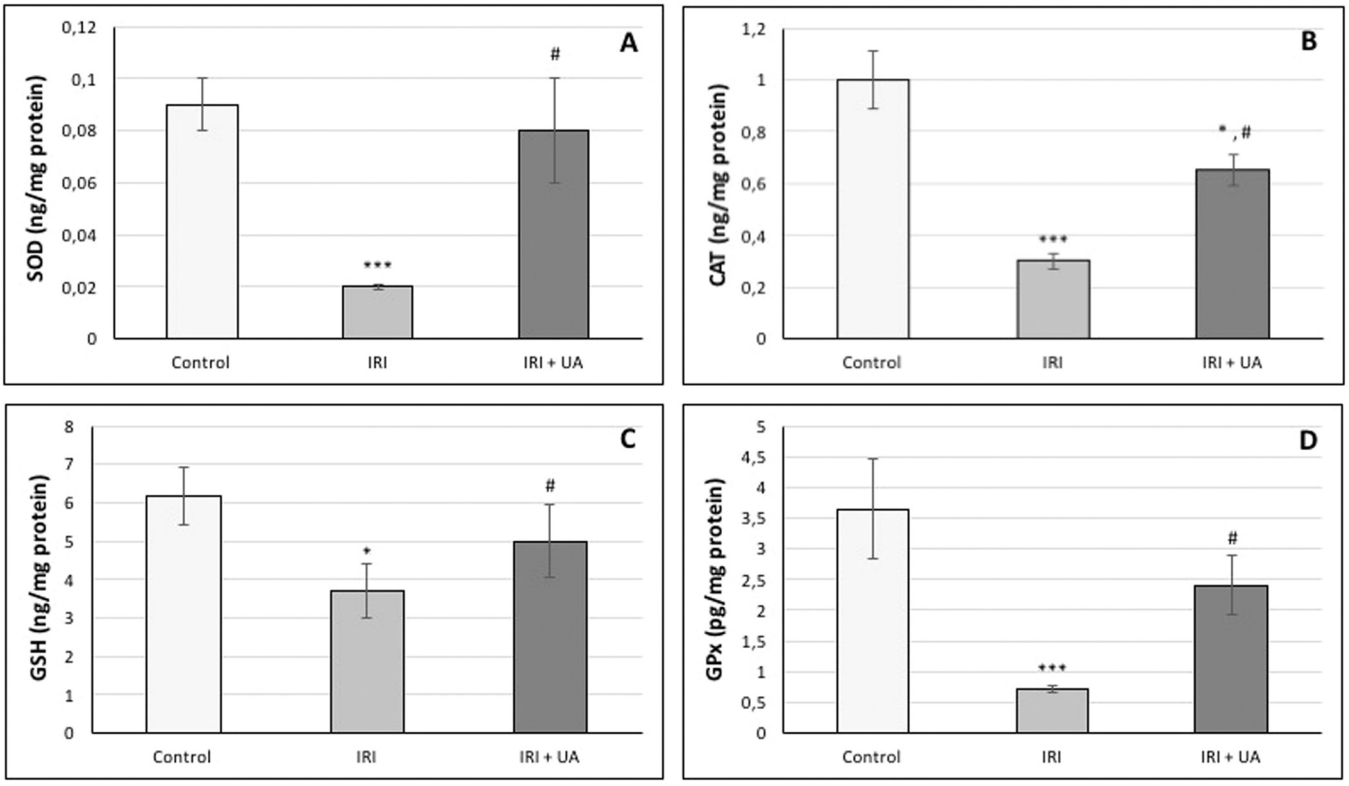

Fig. 2. Effects of treatment with UA on the antioxidant system parameters in testicular IRI (A) SOD, (B) CAT, (C) GSH, (D) GPx. All values are expressed as mean ±SEM. Statistically significant from sham control group *p<0.05 and ***p<0.001. Statistically significant from IRI group #p<0.05.

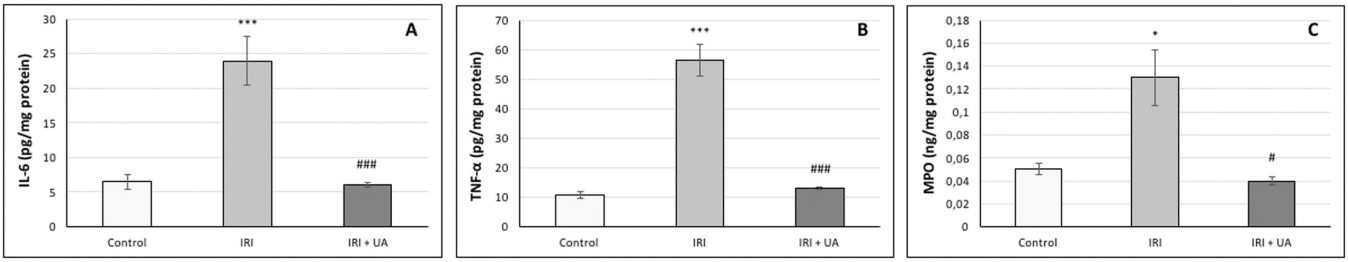

Fig. 3. Effects of treatment with UA on the inflammatory parameters in testicular IRI (A) IL-6, (B) TNF-α, (C) MPO. All values are expressed as mean±SEM. Statistically significant from sham control group *p<0.05 and ***p<0.001. Statistically significant from IRI group #p<0.05 and ###p<0.001.

levels was conducted in accordance with the methodology developed by Mihara and Uchiyama [33]. The absorbance was measured at 532 nm using a microplate reader spectrophotometer (Molecular Devices, CA, USA). The results were expressed in nM/mg protein [34].

The total oxidant capacity (TOC) and total antioxidant capacity (TAC) levels in tissue samples were determined using commercial colorimetric kits (Rel Assay Diagnostics, Gaziantep, Turkey) and oxidative stress index (OSI) was calculated according to the formula given in previous literature [35].

The capacity of the antioxidant system in testicular tissue was determined using commercial ELISA kits (Bostonchem, Boston, MA) by measuring superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) parameters. The coefficient of variation (CV%) for the intra-assay measurements of SOD, CAT, GPx and GSH were determined to be 8.4 %, 7.9 %, 9.0 % and 8.6 %, respectively.

The levels of inflammation in testicular tissue was determined using commercial ELISA kits (Bostonchem, Boston, MA) by measuring interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and myeloperoxidase (MPO) parameters. The CV% for the intra-assay measurements of IL-6, TNF-α and MPO were determined to be 8.0 %, 5.8 % and 7.9 %, respectively.

The levels of ERS and apoptosis in testicular tissue was determined using commercial ELISA kits (Bostonchem, Boston, MA) by measuring heat shock protein family A (HSP70) member 5 (HSPA5, also called as GRP78), activating transcription factor 6 (ATF6), DDIT3 and cleaved caspase-3 parameters. The CV% for the intra-assay measurements of HSPA5, ATF6, DDIT3 and cleaved caspase-3 measurements were determined to be 6.9 %, 4.9 %, 5.4 % and 6.2 %, respectively.

2.5. Statistical analysis

The SPSS 23.0 (Chicago, IL) was used for data analyses. Data were expressed as mean±standard error of the mean (SEM). The Shapiro-Wilk test was employed to assess the normality of the data distribution. One-way ANOVA and post-hoc Tukey test was used to compare the means of groups. $p<0.05$ was considered statistically significant.

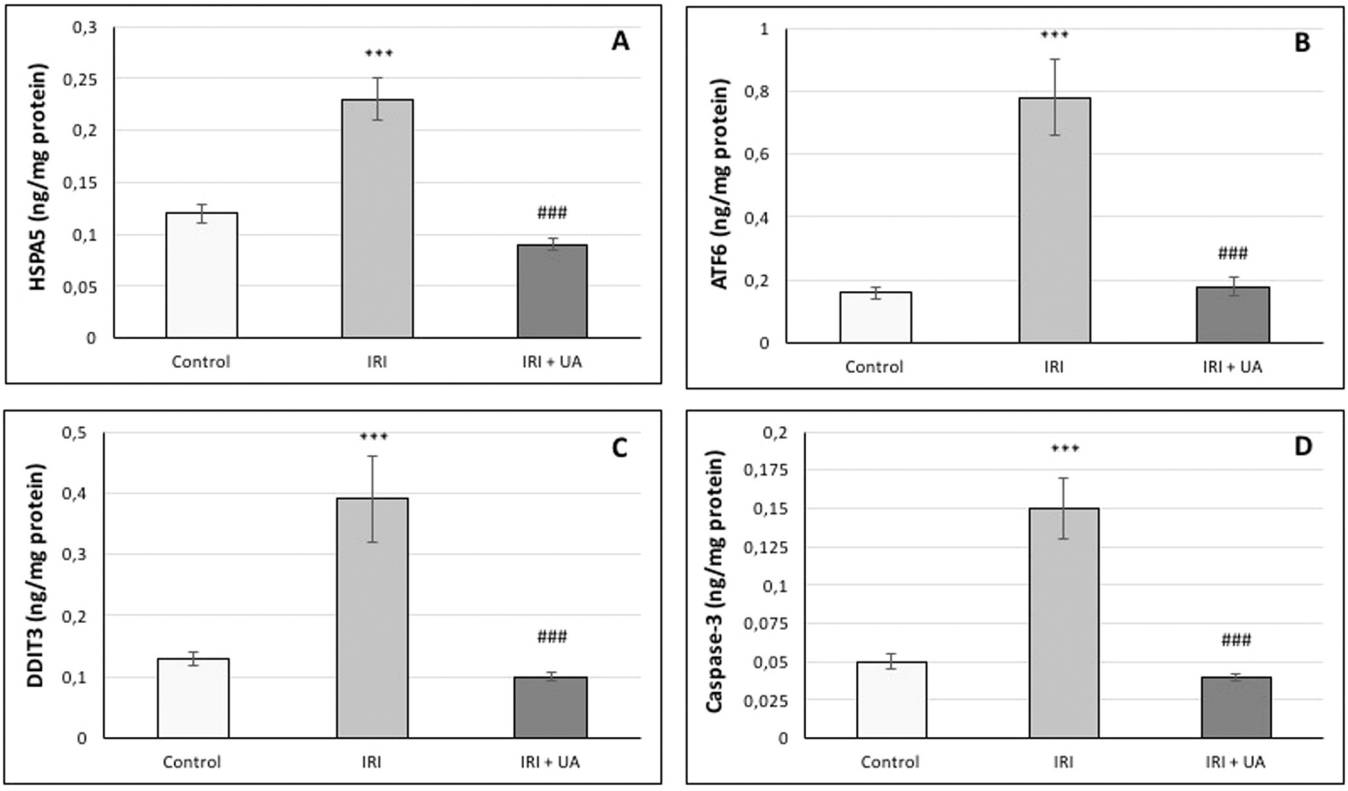

Fig. 4. Effects of treatment with UA on the ERS and apoptosis parameters in testicular IRI (A) HSPA5, (B) ATF6, (C) DDIT3, (D) Caspase-3. All values are expressed as mean±SEM. Statistically significant from sham control group ***p<0.001. Statistically significant from IRI group ###p<0.001.

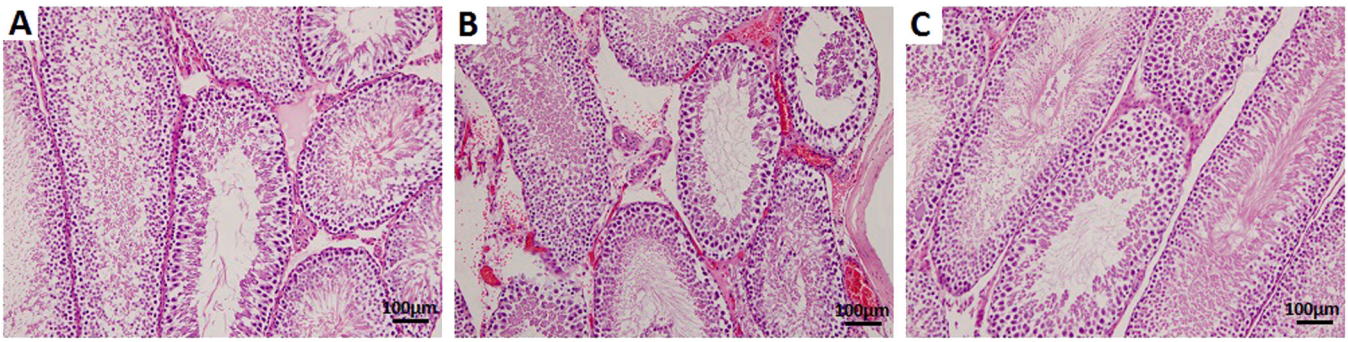

Fig. 5. The testicle tissue sections were stained with H&E and representative photomicrographs were shown in (A) for the sham control group, (B) for the IRI group, and (C) for the IRI+UA group.

3. Results

3.1. The effect of UA on the parameters of the OS in testicular tissue

It is well established that OS plays a pivotal role in the pathogenesis of IRI. Therefore, the effect of UA on I/R-induced testicular OS was examined. As illustrated in Figs. 1 and 2, I/R exposure led to a significant elevation in testicular MDA (163 %), TOC (199 %) and OSI (881 %) values, accompanied by a notable reduction in TAC (57 %), SOD (78 %), CAT (70 %), GPx (81 %) and GSH (40 %) levels in comparison to the sham control group. However, treatment with UA resulted in the regeneration of the testicular antioxidant system, as evidenced by increases in TAC (124 %), SOD (300 %), CAT (117 %), GPx (239 %) and GSH (35 %) levels. Furthermore, the I/R-induced MDA (65 %), TOC (51 %) and OSI (85 %) levels were suppressed.

3.2. The effect of UA on inflammation parameters in testicular tissue

The impact of UA on I/R-induced testicular inflammation was then investigated. As illustrated in Fig. 3, the testicular tissue of rats subjected to I/R exhibited significantly elevated levels of IL-6 (268 %), TNF-α (417 %) and MPO (160 %) in comparison to the sham control group. Conversely, UA treatment was found to significantly suppress IL-6 (75 %), TNF-α (77 %), and MPO (69 %) levels in comparison to the IRI group.

3.3. The effect of UA on ERS and apoptosis parameters in testicular tissue

The impact of UA on I/R-induced testicular ERS and apoptosis was then investigated. As illustrated in Fig. 4, significantly elevated levels of HSPA5 (92 %), ATF6 (388 %), DDIT3 (200 %) and cleaved caspase-3 (200 %) were observed in the testicular tissue of rats subjected to I/R in comparison to the sham control group. Conversely, UA treatment resulted in a significant reduction in the levels of HSPA5 (61 %), ATF6 (77 %), DDIT3 (74 %) and cleaved caspase-3 (73 %) in comparison to the IRI group.

3.4. The effect of UA on testicular pathological changes

The histological analysis conducted on the testicular tissue of animals in the sham control group revealed normal seminiferous tubule morphology with intact germinal epithelium (Fig. 5A). The testicular tissue of rats in the IRI group exhibited degenerative changes in germinal cells, vasocongestion, interstitial oedema, and bleeding (Fig. 5B). Although the testicular tissue of the IRI+UA group rats exhibited degenerative changes and irregularities in the germinal epithelium, its morphology was largely normal in appearance (Fig. 5C).

4. Discussion

TT represents one of the most common urological emergencies in the paediatric age group and the only treatment option is surgical detorsion [7]. It has been demonstrated that both ischemia and detorsion-induced reperfusion can induce testicular damage, which may result in subfertility and infertility [6]. Therefore, research into the potential of molecules with antioxidant and anti-inflammatory properties to prevent testicular damage induced by T/D has gained significant momentum [6,7]. Consequently, the objective of this study was to ascertain the testicular protective effect of UA in an experimental T/D model for the first time. According to the results of the current study; (i) UA reduced testicular IRI by strengthening the antioxidant system in an *in vivo* T/D experimental model. (ii) UA inhibited IRI-induced testicular inflammatory processes. (iii) UA eliminated histopathological findings by reducing ERS and apoptosis. These findings provide support for the hypothesis that UA may have a promising protective role against testicular IRI.

The most preferred method for creating TT in experimental animal models is to rotate the testicle around the spermatic cord at 720° and fix it with 3.0 or 4.0 sutures. Furthermore, it is recommended that the torsion time be between one and four hours to produce acceptable and reversible testicular damage in rats [6]. The longer left spermatic cord structure results in the left testicle being torsioned more frequently than the right testicle [36]. Thus, in order to create an experimental TT model, the left testicle in rats was rotated 720 degrees, resulting in the formation of a testicular IRI model with 4 h of torsion and 2 h of detorsion in this study. Histopathological analysis has a very important place in the identification of IRI [24,26]. The results of our study indicated that the IRI group exhibited increased degenerative changes in germinal cells, vasocongestion, interstitial edema, and bleeding when compared to the sham control group. These findings were consistent with the results of previous reports presenting histopathological findings in experimental models of testicular IRI [24,30,37,38]. Nevertheless, the UA treatment yielded more favourable histopathological findings than those observed in the IRI group. These findings are consistent with previous reports indicating the testicular protective effect of lichen secondary metabolites [39–41].

The pathophysiology of IRI-induced tissue damage is characterised by OS due to an imbalance between ROS and antioxidant capacity. The accumulation of ROS results in the damage of lipids, proteins and DNA [7]. Phospholipids, which constitute the primary structural framework of the cell membrane, are the biomolecules most susceptible to OS [1,38]. The oxidation of lipids, initiated by the action of ROS, results in the formation of reactive aldehyde derivatives, including MDA [34]. TOC, TAC and OSI are common biomarkers of the OS that are frequently employed to ascertain the OS level in tissue or blood samples [26]. The enzymatic antioxidant system, comprising SOD, CAT and GPx, protects cells against ROS-related damage by catalysing the gradual reduction of the superoxide radical to water [27,34]. GSH is the most important

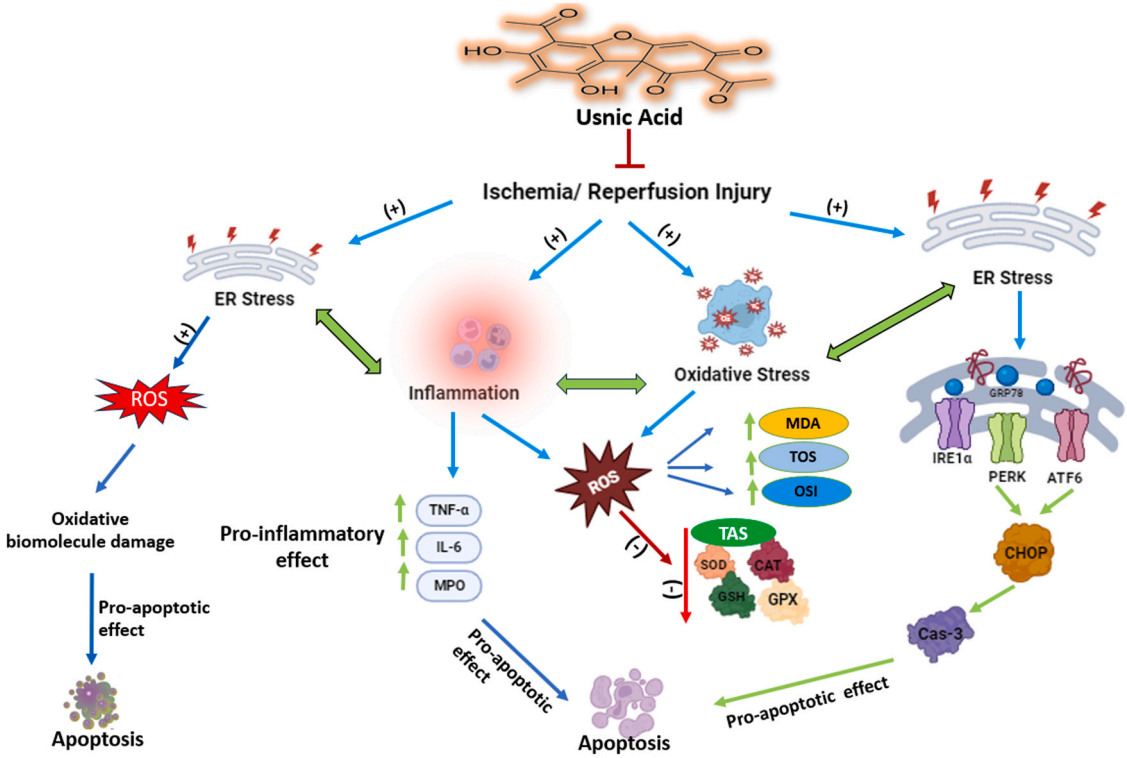

Fig. 6. A schematic summary of the biochemical mechanisms underlying the protective effect of UA in a testicular IRI model (created using https://www.biorender.com).

endogenous non-enzymatic antioxidant molecule [28]. The current study corroborated previous reports [26,36–38] by demonstrating that OS levels are elevated and the antioxidant system is suppressed in the T/D model. Nevertheless, UA treatment enhanced the resilience of the antioxidant system, enabling the testicular OS to be tolerated. These findings were considered to provide evidence of *in vivo* antioxidant activity of UA against testicular IRI. It has been demonstrated that the antioxidant properties of UA are due to its ability to donate electrons, scavenge ROS and chelate metal ions [16,19]. Surprisingly, it is noteworthy that the ROS scavenging activity of UA has been demonstrated to be comparable to that of phenolic acids, including caffeic and sinapic acid [42]. Furthermore, various *in vivo* studies have demonstrated that UA facilitates the removal of ROS by enhancing the activity of antioxidant enzymes [22,27–29].

Inflammation is a protective response of the body to external stimuli. It serves to prevent irreversible damage by initiating the repair mechanisms of tissue damage [43]. Once homeostasis is achieved, inflammation is eliminated in a controlled manner. However, in the event that anti-inflammatory mechanisms are ineffective, inflammation becomes chronic, which in turn results in further tissue damage [44]. Experimental studies have demonstrated that the uncontrolled increase in the levels of ROS as a consequence of I/R not only elevates OS but also stimulates the release of pro-inflammatory cytokines by activating nuclear factor-kappa B, a pivotal transcription factor that regulates the development of inflammatory tissue damage [1,3]. During the inflammatory process, neutrophils are directed to damaged tissue areas and attempt to protect the host tissue through the secretion of enzymes, including MPO. Consequently, an elevation in MPO levels is a distinctive indicator of tissue inflammation [27]. In accordance with previous reports [6,7,26,38], the current study demonstrated that the levels of inflammation were elevated in the IRI group. Nevertheless, the levels of inflammatory biomarkers were found to be suppressed by UA treatment in this study. This indicated that UA alleviates testicular IRI through its anti-inflammatory properties, which are mediated by its antioxidant activity. These results were in accordance with previous reports which had demonstrated the anti-inflammatory effect of UA in experimental models of gastric, lung, and brain injury induced by inflammation [27,29,45].

It is well established that I/R-induced OS and inflammation can lead to ERS, mitochondrial dysfunction and apoptosis [11,46]. The increased of ERS results in the activation of the UPR pathway within the cell. The cells contain three sensors that detect the UPR: protein kinase RNA-like ER kinase, inositol-requiring enzyme type 1 and ATF6 [47]. These sensors are in an inactive state due to their interaction with HSPA5, a chaperone, under physiological conditions [9]. The activation of the UPR strives to alleviate the burden of unfolded or misfolded proteins by dissociating HSPA5 from sensor proteins in the lumen [10]. Following its release, ATF6 is transported into the Golgi apparatus, where it is cleaved to form an active transcription factor. Following its activation, ATF6 is transported to the nucleus, where it induces the expression of multiple UPR pathway proteins, including HSPA5, HSP90B1 and DDIT3 [11]. In the event that ER homeostasis is not restored as a consequence of UPR activation, the elevated expression of DDIT3 induces caspase-dependent apoptosis [12]. Our findings align with those of previous studies [10–12], indicating that ERS and apoptosis levels were elevated in the IRI group relative to the sham control group. Nevertheless, the results demonstrated that UA treatment could effectively mitigate the elevated levels of ERS and apoptosis. The data obtained were consistent with previous findings indicating that UA can reduce ERS and apoptosis in experimental models [20,21,47]. Nevertheless, the precise mechanism by which UA inhibits ERS in testicular IRI remains unclear. It is postulated that this is due to the antioxidant activity of UA, which has been previously demonstrated and confirmed in this study [16,18,19].

Our research also has a few limitations. Firstly, this study was able to include the protective effect of a single dose of UA. Secondly, the protective effectiveness of UA was evaluated prior to reperfusion, but there is currently no information available regarding its effectiveness following reperfusion. Thirdly, the molecular mechanisms underlying the testicular protective effect of UA require further investigation.

Fourth, although biochemical and histological improvements have been demonstrated in rats treated with UA, it is necessary to investigate whether UA restores the reproductive functions of rats.

5. Conclusion

The findings of this study indicate that UA may have the potential to suppress the inflammatory response, OS, ERS and apoptosis that are induced by I/R in the testicular tissue (Fig. 6). These results provide new information about the protective mechanism of UA against testicular IRI and indicate that UA is a promising choice in the treatment of ischemic testicle. However, this study did not confirm the effect of UA on testicular IRI-induced reproductive potential change; this should be examined in more detail in a follow-up study.

Ethics approval

This study was approved by the Animal Experiments Local Ethics Commitee of Karadeniz Technical University (Protocol no: 2022/43) and performed according to the animal research reporting of *in vivo* experiments (ARRIVE) guidelines.

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CRediT authorship contribution statement

Selim Demir: Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft. **Ilke Onur Kazaz:** Conceptualization, Methodology, Writing – review & editing. **Sevdegul Aydin Mungan:** Methodology, Investigation. **Nihal Turkmen Alemdar:** Investigation, Formal analysis. **Nijazi Perolli:** Methodology, Investigation. **Elif Ayazoglu Demir:** Investigation, Formal analysis. **Ahmet Mentese:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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