Original Article

Renoprotective Potential of the Ultra-Pure Lipopolysaccharide from Rhodobacter Sphaeroides on Acutely Injured Kidneys in an Animal Model

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Abstract

One of the main causes of acute kidney injury is ischemic reperfusion injury (IRI). Inflammatory response, apoptotic damages, and oxidative stress-related injuries are all involved in the pathogenesis of IRI. Toll-like receptors (TLR) are strongly associated with IRIs, especially TLR4, which is markedly induced in response to IRI. Accordingly, the current study aimed to investigate the potential renoprotective effect of ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (ULPS-RS) at two doses in an animal model of bilateral IRI. A total of 30 adult male rats were divided randomly into five equal groups of control (laparotomy plus bilateral renal IRI), vehicle (same as the control group, but pretreated with the vehicle), sham (laparotomy only), ULPS-RS (same as the control group, but pretreated with 0.1 mg/kg of ULPS-RS), and ULPS-RSH (same as the control group, but pretreated with 0.2 mg/kg of ULPS-RS). Subsequent to 30 min of ischemia and 2 h of reperfusion, serum samples were collected for measuring urea, creatinine, and neutrophil gelatinase-associated lipocalin. Afterward, tissue samples were obtained from all animals to measure inflammatory mediators (interleukin 6, interleukin 1β, and tumor necrosis factor α), oxidative stress marker (8-isoprostane), apoptosis mediators (B cell lymphoma 2 [Bcl2]), and Bcl2-associated X protein (Bax). In the control group, all of the measured parameters were significantly elevated in response to IRI, except for Bcl2, which decreased significantly. On the other hand, exactly opposite effects were observed in the ULPS-RS treated groups indicating the nephroprotective effect of this compound against IRI at both tested doses. The findings reveal for the first time that ULPS-RS has the therapeutic potential of attenuating the renal dysfunction induced by IRI.

Keywords: Bax; Bcl2; F2-isoprostane; IL-1β; IL6; Ischemic reperfusion injury; NGAL; pure TLR4 antagonist; TNFα

1. Introduction

Ischemia-reperfusion injury (IRI) of the kidney is a critical medical condition that is characterized by the sudden impairment of blood flow to the kidneys, followed by the restoration of blood flow and re-oxygenation (1). Among the common causes of renal IRI are partial nephrectomy, surgery involving aorta clamping, shock, sepsis, trauma, and renal transplantation (2). In particular, in renal transplantation, the IRI damage could cause graft dysfunction and rejection, leading to serious postoperative complications and even death (3). Furthermore, IRI is one of the major causes of morbidity and mortality, which represents an important progressive risk factor for chronic kidney disease (4, 5).

IRI involves two phases including the dysfunction of the glomerular capillary endothelial layer along with the necrosis of the tubular epithelial cells, precipitated

by ischemia, followed by the production of reactive oxygen species during reperfusion. In fact, these two phases initiate a robust inflammatory reaction (6). Unfortunately, the resulting reperfusion exacerbates the injury via inducing several mechanisms, including the innate and the adaptive immune responses and even the cell death programs (7). More importantly, reperfusion stimulates nitric oxide synthase and increases its expression, thereby increasing nitric oxide levels which eventually augment tissue injury through lipid peroxidation, DNA damage, and proapoptotic effects (8). Additionally, since inflammation in IRI results in more renal damages, it is essential to prevent it for protecting the area (9). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin 6 (IL-6), have a primary role in renal IRI (10). On the other hand, the signals liberated from the dying cells induce the Toll-like receptors (TLRs), mainly TLR4, which encodes the genes regulating the inflammatory cells and the mediators (11). When activated, TLRs activate the production of proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, and IL-6 (12). They also involved the initiation of apoptosis in renal IRI (13). The process of apoptosis is an important mechanism in the course of renal IRI, and it has been reported to be associated with increased levels of proapoptotic Bcl2-associated X protein (Bax)and reduced levels of the apoptosis inhibitor B cell lymphoma 2 (Bcl2) (14).

As described previously, the pathophysiology of the kidney IRIs is complicated and there are multiple targets to be manipulated for attenuating the resulting kidney injury. However, successful and effective treatment is still unavailable (15). An important emerging therapeutic target for IRI is TLRs, and numerous studies have revealed the essential role of TLRs in IRI (3). Both glomeruli and renal tubular epithelial cells have been found to express TLR1, TLR2, TLR3, TLR4, and TLR6 (16). Additionally, TLR-4 increasingly affects the pathogenesis of renal IRI (17). Moreover, Wu et al. demonstrated that TLR4 was upregulated in tubular epithelial cells after any IRI in the kidney. They also

reported that the deficiency of TLR4 reversed the IRI-stimulated release of pro-inflammatory cytokines and chemokines, and also inhibited the accumulation of macrophages and neutrophils (18). Furthermore, it has been found that antagonizing this receptor by TAK-242 results in attenuating IRI (19).

Several studies have tested ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (ULPS-RS) as a selective TLR4 (20). However, to the best of our knowledge, very few, if any, studies have tested ULPS-RS with regard to IRI; nonetheless, previous research has shown that ULPS-RS can reduce cerebral vasospasm in the animal model of subarachnoid hemorrhage (21). Accordingly, it is hypothesized in the present study that ULPS-RS will, through the modulation of TLR4, have a nephroprotective effect in the IRI rat model induced by bilateral ligation of renal pedicles.

2. Materials and Methods

2.1. Experimental Animals

A total of 30 male Wister Albino rats with the age of more than 20 weeks and a mean weight of 300±50 g were selected for this study. The rats were kept at an animal house in the Animal Resources Centre of the College of Sciences, Kufa University, Kufa, Iraq. All rats had free access to food and water; moreover, they were accustomed to a controlled temperature (25°C) and humidity (60%-65%) and were kept in their cages under 12-h cycles of light and dark.

2.2. Animal Experiment

Bilateral IRI of the kidneys was induced as previously reported (22). Briefly put, animals were anesthetized with an injection of ketamine (100 mg per kilogram of body weight) and xylazine (10 mg per kilogram of body weight) intraperitoneally (23). Subsequently, the abdominal area was shaved and an incision was made. Subsequently, the renal pedicles were clamped bilaterally with non-traumatic vascular clamps for 30 min. About one milliliter of normal saline was given into the abdominal area, followed by covering the incision sites with moist gauze to keep the animals'

well-hydrated status during the experiment (24). Following that, the clamps were removed, and the reperfusion period started, which continued for 2 h. In the next stage, a cardiac puncture was performed to collect blood samples, and finally, after scarifying the animals, the kidneys were immediately collected (25).

2.3. Study Design

Animals were randomly divided into five groups of six rats per group. The groups included sham (negative control, surgical procedures similar to the control group without clamping of the renal pedicles), control (positive control, IRI group), vehicle (similar to the control group, but preinjected with pyrogen-free distilled water), ULPS-RS (similar to the control group, but preinjected with ULPS-RS at a dose of 0.1 mg/kg body weight) (26), and ULPS-RSH (similar to the control group, but preinjected with 0.2 mg/kg body weight of ULPS-RS. ULPS-RS was ordered from InvivoGen, USA. The compound was reconstituted in endotoxin-free distilled water and injected intraperitoneally to the ULPS-RS groups one hour before the IRI induction (27).

2.4. Sample Collection

2.4.1. Serum Samples

Blood was collected by cardiac puncture, placed into gel tubes without anticoagulant, and allowed to clot for 20 min. Centrifugation was performed for 10 min at 3000 rpm in order to collect serum samples (28). The collected serum samples were then used for the determination of urea, creatinine, and neutrophil gelatinase-associated lipocalin levels for the assessment of kidney function.

2.4.2. Tissue Samples

Following the collection of kidney samples, the remaining parts of the kidneys were washed with cold phosphate-buffered saline (PBS) several times to remove residual blood clots or cells. Subsequently, the tissues were homogenized (1:10 w/v) in cold lysis buffer (PBS at pH=7.4, 1% of Triton-X 100, and protease inhibitor cocktail). The homogenization was conducted using a high-intensity liquid processor (29). The homogenates

were then placed in a cold centrifuge at $4°C$ with a speed of 4000 rpm as specified by the enzyme-linked immunosorbent assay (ELISA) kits' instructions. Afterward, the supernatants were transferred into different aliquots and kept in the deep freeze, which were utilized later for determining the concentrations of neutrophil gelatinase-associated lipocalin (NGAL), IL-6, IL-1β, TNF-α, and 8-isoprostane using ELISA. All ELISA kits were purchased from a company called Bioassay Technology Laboratory.

2.5. Statistical Analysis

Statistical analysis was performed using SPSS software (version 21) through one-way analysis of variance, followed by Tukey post hoc test for multiple comparisons among the experimental groups. A P-value less than 0.05 was considered statistically significant in analyzing the obtained results.

3. Results

3.1. ULPS-RS Ameliorates Renal Function

Statistical analysis revealed significant elevations in the serum levels of urea, creatinine, and NGAL, compared to the sham group ($P<0.05$). On the other hand, serum levels of these parameters significantly reduced ($P<0.05$) in response to ULPS-RS treatment at both doses shown in figures 1, 2, and 3. These findings indicate that ULPS-RS can improve renal function by reducing the aforementioned parameters.
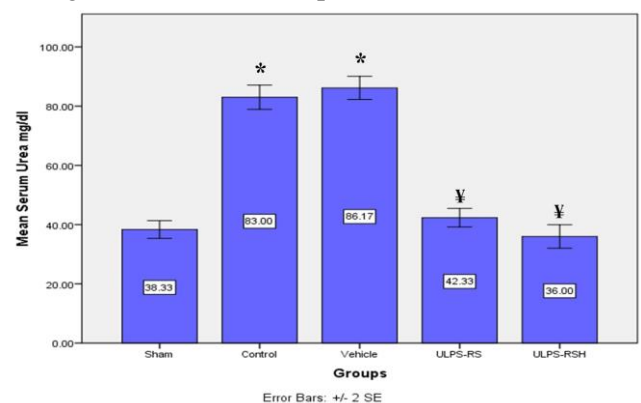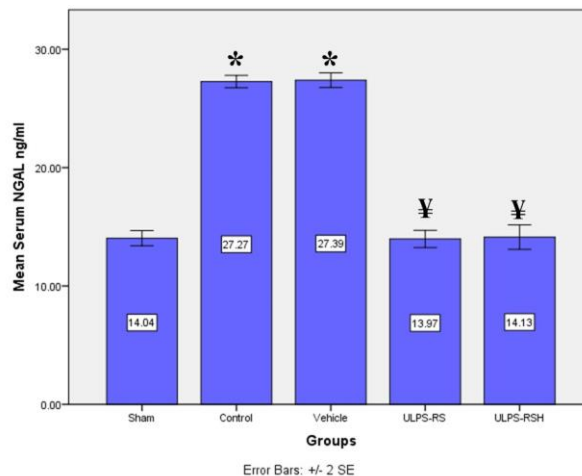

Figure 1. Mean urea serum levels in the experimental groups (n=6 in each group)
* $P<0.05$, compared to the sham group, ¥ $P<0.05$, compared to the control group
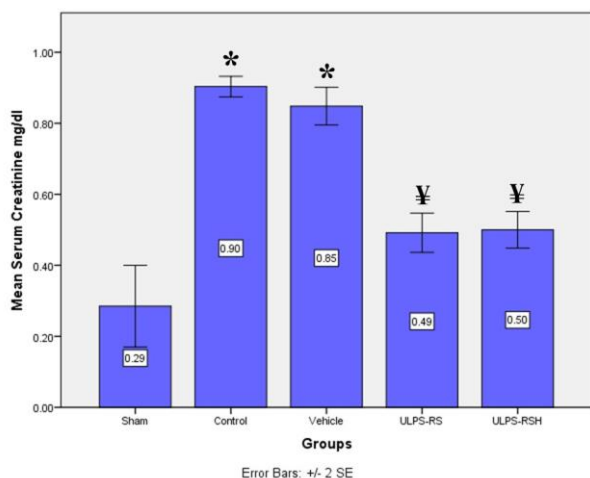
Figure 2. Mean creatinine serum levels in the experimental groups (n=6 in each group)
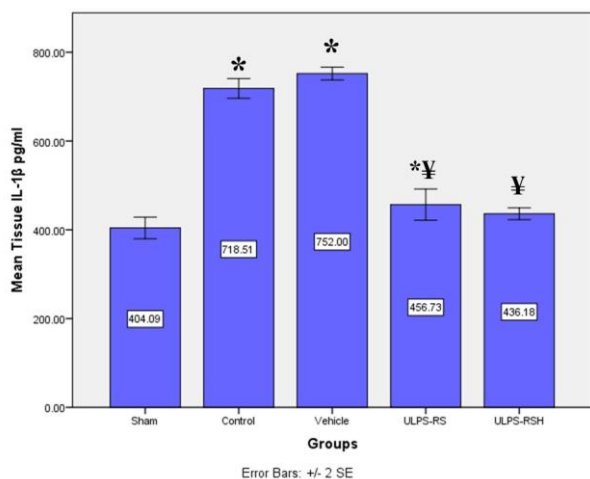* $P<0.05$, compared to the sham group, ¥ $P<0.05$, compared to the control and sham groups

Figure 3. Mean NGAL serum levels in the experimental groups (n=6 in each group)
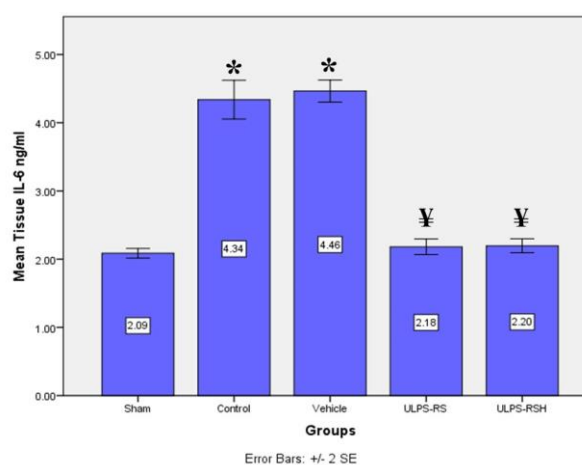* $P<0.05$, compared to the sham group, ¥ $P<0.05$, compared to the control group

3.2. ULPS-RS Possesses an Anti-Inflammatory Effect

The present study shows that the renal tissue levels of IL-1β (Figure 4), IL-6 (Figure 5), and TNF-α (Figure 6) significantly elevated in response to IRI in the control and vehicle groups in comparison with the sham group ($P<0.05$). A significant reduction ($P<0.05$) in the tissue levels of the inflammatory mediators was also observed in both treatment groups (i.e., ULPS-RS and ULPS-RSH), compared to the control group. All of these parameters have comparable levels in the sham and treatment groups. However, the IL-1β levels followed dose-dependent effect, which was comparable to the sham group only at the higher dose used in the ULPS-RSH group.

Figure 4. Renal tissue levels of IL-1β in the five investigated groups (n=6 in each group)
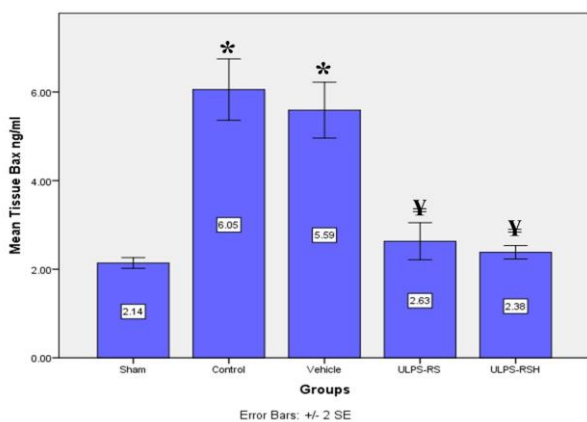* $P<0.05$, compared to the sham group, ¥ $P<0.05$, compared to the control group

Figure 5. Renal tissue levels of IL-6 in the five investigated groups (n=6 in each group)
* $P<0.05$, compared to the sham group, ¥ $P<0.05$, compared to the control group
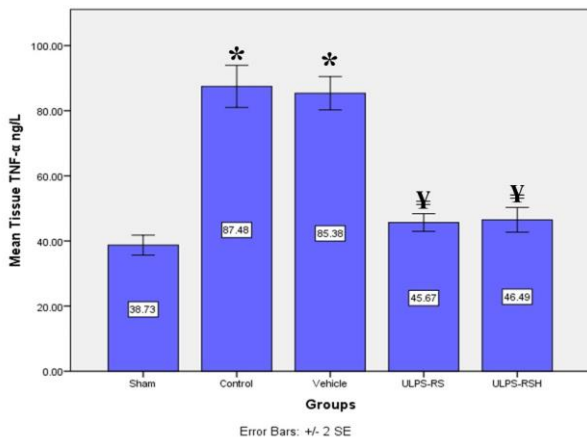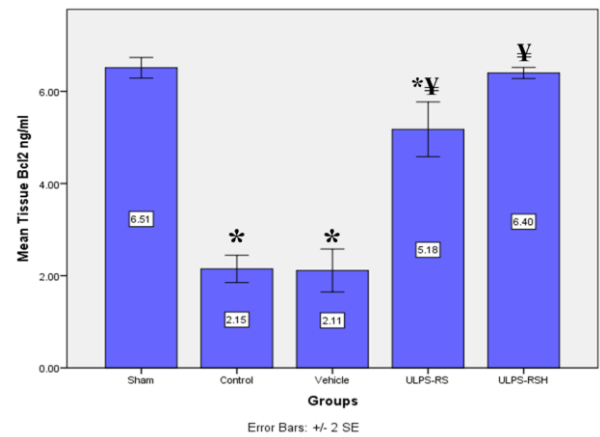
Figure 6. Renal tissue levels of TNF-α in the five investigated groups (n=6 in each group)
* *P*<0.05, compared to the sham group, ¥ *P*<0.05, compared to the control group

Figure 8. Renal tissue levels of Bcl2 in the five investigated groups (n=6 in each group)
* *P*<0.05, compared to the sham group, ¥ *P*<0.05, compared to the control group

3.3. ULPS-RS Exerts an Anti-Apoptotic Effect

Based on the findings of this study, renal tissue levels of Bax were significantly higher, and those of Bcl2 were significantly lower in the control and vehicle groups, respectively, compared to the sham group (*P*<0.05). An opposite effect was observed in the treatment groups in that the tissue levels of Bax were significantly lower, and Bcl2 was significantly higher in the ULPS-RS and ULPS-RSH groups, compared to the control group (*P*<0.05). Furthermore, only the ULPS-RSH group showed an insignificantly different Bcl2 level from the sham group as shown in figures 7 and 8. Accordingly, it is concluded that ULPS-RS can decrease the Bax/Bcl2 ratio, and consequently, inhibit apoptosis.

3.4. ULPS-RS Shows an Anti-Oxidant Effect

Statistical analysis of ELISA results exhibited marked elevation in oxidative stress as manifested by the significantly higher levels of 8-isoprostane in the control and vehicle groups, compared to the sham group (*P*<0.05). Interestingly, ULPS-RS demonstrated an anti-oxidant potential by significantly reducing renal tissue levels of 8-isoprostane at both doses in comparison with the control group as shown in figure 9.

Figure 7. Renal tissue levels of Bax in the five investigated groups (n=6 in each group)
* *P*<0.05, compared to the sham group, ¥ *P*<0.05, compared to the control group
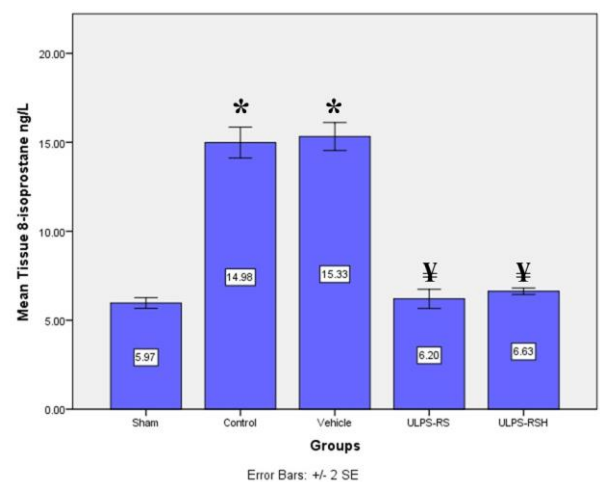
Figure 9. Renal tissue levels of 8-isoprostane in the five investigated groups (n=6 in each group)
* *P*<0.05, compared to the sham group, ¥ *P*<0.05, compared to the control group

4. Discussion

The current study revealed for the first time that ULPS-RS has a nephroprotective effect as manifested by the reduction in the serum levels of urea, creatinine, and NGAL. This effect could partly be attributed to the anti-inflammatory activity shown by ULPS-RS as reflected in the reduction of the inflammatory mediators of IL-1β, IL-6, and TNF-α. An additional mechanism behind the observed renoprotective effect is the marked reduction in the oxidant stress biomarker 8-isoprostane in both ULPS-RS treated groups.

4.1. Animal Model of Renal IRI

The animal model of IRI used in this study is a well-established model that has been extensively studied as an *in vivo* model of acute renal injury. It is also well-documented that the 30 min of bilateral renal ischemia was accompanied by significant and acute kidney injuries (24, 30). Additionally, renal dysfunction after two hours of reperfusion has been shown to be intensive and associated with oxidative stress-related damages in renal tissues (22). Male rats were chosen for the study because it has been shown that they were more susceptible to renal IRI (31).

4.2. Effect of ULPS-RS on Renal Function Parameters

In this study, serum urea and creatinine were measured as markers of excretory renal function, and NGAL was measured as a marker of structural kidney damage. The serum levels of urea and creatinine significantly elevated after IRI in the control group, indicating the initiation of renal dysfunction. This is in line with previous attempts confirming that both parameters increase after 2 h of ischemia (32). On the other hand, ULPS-RS at both tested doses significantly reduced the urea and creatinine levels, which is an indication of a nephroprotective effect against the renal IRI. The observed reduction in the serum levels of urea and creatinine levels confirms the results of previous studies which investigated the potential renoprotective effect of eritoran, another TLR4 antagonist (33).

Regarding NGAL, the control group exhibited significant upregulation in response to the induction of renal IRI. NGAL is an accurate and reliable biomarker of acute renal tubular injury that increased markedly only two hours after acute renal injuries (34). The significantly reduced NGAL levels, which are demonstrated in the ULPS-RS and ULPS-RSH groups, compared to the control non-treated groups, suggest that ULPS-RS has a protective effect on the renal structural damage induced by IRI. These results were in agreement with the findings of previous research that reported the renoprotective effect of the TLR4 antagonist TAK-242 against renal IRI in the animal model, an effect that was accompanied by the reduction of plasma NGAL levels (19).

4.3. Effect of ULPS-RS on Inflammatory Mediators
4.3.1. Effect of ULPS-RS on IL-1β

The significantly elevated levels of IL-1β found in the control groups are in line with the results of a study by Bivol, Iversen (35) study. Different cells, including the renal parenchymal cells, have been reported to produce IL-1β. IL-1β is one of the downstream effector molecules released after TLR4 stimulation and was documented to be crucially involved in the pathogenesis of the IRI and the potentiation of the inflammatory response in acutely injured tubular cells of the kidneys (12, 36). Accordingly, blocking TLR4 with eritoran has been found to be renoprotective, an effect that was partly mediated through reducing IL-1β levels (33), which confirms the findings of the present study in that the protective effect of ULPS-RS was also associated with significantly reduced IL-1β tissue levels, compared to the control group.

4.3.2. Effect of ULPS-RS on IL-6

Bivol, Iversen (35) have found IL6 to elevate within the kidney immediately and systemically following the ischemic insult, which holds close similarity with the present study in which there was a significant elevation in IL6 in the control group, compared to the sham group. IL6 is another cytokine within the cascade of inflammation that is able to directly harm the kidney cells (37). The sources of IL6 in the kidneys include podocytes, endothelial, and mesangial along with the renal tubular epithelial cells (38). Through stimulating

the inflammatory pathway, TLR4 has been reported to be directly involved in elevating IL6 (39). This gives a reasonable explanation for the significant decrease in IL6 found in this study after treating the animals with the TLR4 antagonists, compared to the control groups (i.e., LPS-RS and ULPS-RS). This means that the renoprotective effect of ULPS-RS is mediated partly by the reduction in IL6 levels.

4.3.3. Effect of ULPS-RS on TNF-α

As the findings of this study suggest, TNF-α was significantly elevated in the control and vehicle groups, compared to the sham group. Previous research has shown similar results in that TNFα levels in renal tissues more significantly elevated after an hour of both ischemia and reperfusion, an increment that persisted for 4 h following reperfusion (40). Other studies have also found renal IRI to be accompanied by high production of inflammatory cytokines, such as TNFα and IL6 (41). TNFα is a proinflammatory cytokine released by various cells, such as B- and T-cells along with other cells, including cardiac myocytes, endothelial cells, and glomerular mesangial cells (42). Both the MAP kinases induced by the injury and the oxidants liberated after the reperfusion phase were found to be responsible for the synthesis of TNFα. Moreover, TNFα itself augments its own release by a positive feedback loop (43). On the other hand and in agreement with previous studies, TNFα levels in the ULPS-RS and ULPS-RSH groups were significantly reduced, compared to the control groups (33).

4.4. Effect of ULPS-RS on the Apoptosis Mediators

There were significantly higher renal tissue concentrations of BAX and lower Bcl2 levels in the control and vehicle groups, compared to the sham group. Apoptosis, which occurred within 2 h after reperfusion, is a major event during the genesis of renal IRI (44). Both intrinsic and extrinsic apoptosis pathways occur during IRI and in the epithelial cells of the kidneys. In fact, Bax is a primary proapoptotic protein induced by an ischemic stimulus which can attack the mitochondria stimulating apoptosis.

However, the action of Bax is counteracted by anti-apoptotic Bcl2 protein which blocks the effect; however, it reduces early after ischemia (45). In general, ischemia increases Bax/Bcl2 ratio, and therefore, initiates apoptosis (46).

Following the treatment, Bax significantly reduced, and Bcl2 significantly increased in both ULPS-RS groups, compared to the control group and even with the sham group with higher a dose of ULPS-RS. This suggests that ULPS-RS inhibits apoptosis, and accordingly, provides a nephroprotective effect against IRI. Consistent with these results, studies have demonstrated that TLR4 activation can decrease Bcl2 expression and directly activate caspase 8, while TLR4 blockade decreases Bax expression (17, 47).

4.5. Effect of ULPS-RS on 8-isoprostane

F2-Isoprostanes are isomers of prostaglandin F2α produced non-enzymatically after the peroxidation of arachidonic acid by free radicals (48). Isoprostanes have been found to be synthesized by the kidneys' glomerular endothelial and mesangial cells (49). There are four classes of the F2-isoprostanes; however, the majority of studies have mainly focused on 8-isoprostane, which is also called 8-isoPGF2α, iPF2α-III, and 15-F2t-ISoP (50). The 8-isoprostane was measured in this study as one of the most reliable oxidative stress markers (51).

There are multiple sources of oxidative stress during the IRI, including the hypoxia-inhibitory factor (1α), which is stimulated during hypoxia, and the nitric oxide synthase, which ultimately leads to the production of reactive oxygen species. Furthermore, oxidative stress is augmented during the reperfusion phase by the mitochondria along with the activation of NADPH oxidases (52). Neutrophils are another important source of reactive oxygen species, which also accumulate immediately after the reperfusion within the renal tissues (53). Furthermore, the cytokines released during IRI also have a role in increasing oxidative stress (54).

Accordingly, the renal tissue levels of 8-isoprostane were significantly elevated in the control group,

compared to the sham group. On the other hand, renal tissue levels of 8-isoprostane reduced significantly in the ULPS-RS and ULPS-RSH groups, compared to the control group and were comparable to the sham group. This indicates that the ULPS-RS has an antioxidant effect which could partly mediate the renoprotective effect observed in these groups. Similar results with other TLR4 inhibitors have also been reported (19). However, further investigation is required to note whether this was a direct effect of ULPS-RS or it was influenced by the reductions in the measured inflammatory mediators in this study.

In conclusion, our findings provide strong evidence that ULPS-RS, at both doses, provide significant protection against renal damages induced by the IRI, an effect associated with the improvement of renal function, an anti-inflammatory effect via reducing the inflammatory cytokines, an anti-apoptotic effect, and an anti-oxidant effect through the reduction of oxidative stress biomarker. Therefore, ULPS-RS could provide a useful preventive treatment against the renal injuries associated with IRI.

Authors' Contribution

Study concept and design: A. R. A. R.

Acquisition of data: M. S. A.

Analysis and interpretation of data: N. R. H.

Drafting of the manuscript: A. R. A. R.

Critical revision of the manuscript for important intellectual content: M. S. A.

Statistical analysis: M. S. A.

Administrative, technical, and material support: M. S. A.

Ethics

All experimental and animal housing procedures were supervised by the Institutional Animal Care and Use Committee in the University of Kufa, Kufa, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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