

Original Article

Effect of Lipopolysaccharide from *Rhodobacter sphaeroides* on Inflammatory Pathway and Oxidative Stress in Renal Ischemia/Reperfusion Injury in Male Rats

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Abstract

Ischemia/reperfusion injury (IRI) is caused by a sudden temporary impairment of the blood flow to the particular organ. The IRI of the kidneys is one of the main causes of acute kidney injury. A vigorous inflammatory and oxidative stress response to hypoxia and reperfusion usually happens as IRI consequences that disturb the organ function. The current study aimed to investigate the effect of antagonizing toll-like receptors (TLRs) effects by lipopolysaccharide obtained from *Rhodobacter sphaeroides* (LPS-RS) on this critical condition. In total, 28 adult male Wistar rats were divided into four groups (n=7) as follows: the sham group which underwent only laparotomy; control group that underwent laparotomy and IRI induction; vehicle group which was similar to the control group plus vehicle treatment, LPS-RS group that was similar to the control group but was pretreated with 0.5 mg/kg of LPS-RS. The results of the current research showed that LPS-RS reduced interleukin-1 β , interleukin-6, tumor necrosis factor α , and 8-isoprostane levels, compared to the control IRI group. However, LPS-RS did not ameliorate the kidney injury as manifested by the elevated levels of urea, creatinine, and neutrophil gelatinase-associated lipocalin. Taken together, the present study demonstrated that LPS-RS at the tested dose failed to offer a renoprotective effect against the IRI in rats.

Keywords: F2-Isoprostane, Il-6, Il-1 β , Lps-Rs, Neutrophil Gelatinase Associated Lipocalin, Ngal, Renal Ischemic Reperfusion Injury, TLR2, TLR4, TNF- α

1. Introduction

An unavoidable consequence of surgical procedures that involve clamping of the aorta and renal transplantation is the ischemic reperfusion injury (IRI) of the kidneys (1). The IRI is critically involved in mediating renal graft dysfunction (2). Additionally, IRI is one of the main causes of acute kidney injury that affects approximately 13 million of the worldwide population (3). The IRI of the kidneys is a critical condition that is manifested by sudden reduction followed by restoration of blood flow to the kidneys (4).

The pathophysiological mechanism underlying renal IRI is extremely complicated. In short, the ischemic phase induces dysfunction of the capillary endothelial cells of the glomeruli and necrosis of the renal tubular epithelial cells. Afterward, the reperfusion phase exacerbates the damage by activating different enzymes with the release of huge amounts of reactive oxygen species and in both phases, a robust inflammatory response is involved (5). Additionally, both the innate and adaptive immune responses are involved in the IRI as well as different cell death programs (4).

An important target for treating renal IRI is the toll-like receptors (TLRs), especially TLR2 and TLR4 that have been extensively studied for their pivotal roles in the genesis of IRI (6). Both receptors were documented to be activated in renal IRI and ultimately participate in releasing different proinflammatory cytokines, such as interleukin (IL)-1 β , IL6, and tumor necrosis factor (TNF)- α (7).

Lipopolysaccharide (LPS) from *Rhodobacter sphaeroides* (LPS-Rs) is a potent TLR4 antagonist in both human and murine cells and prevents TLR4 mediated inflammation. The LPS-Rs is a Penta-acylated lipid A and utilizes two distinct mechanisms to block LPS/TLR4 signaling. The mechanism involves direct competition between under acylated lipid A and hexa-acylated lipid A for binding on MD-2. However, another mechanism implies the ability of penta-acylated lipid A:MD-2 complexes to inhibit hexa-acylated endotoxin:MD-2 complexes and TLR4 functions. Inhibition of TLR4 signaling is possible by the antagonist. Therefore, the current study aimed to investigate the effect of LPS-RS on the treatment of renal IRI in an animal model with bilateral IRI since LPS-RS has been proposed as an antagonist of TLR2 and TLR4 in a previous study (8).

2. Material and Methods

2.1. Experimental Animals and Ethics

This study was performed on 28 male Wister Albino rats weighing 250-350 g. All the animals had free access to food and water and were subjected to a 12:12 light-dark cycle. The temperature and humidity were controlled at 25 °C and 60-65%, respectively. This study was ethically approved by the Institutional Animal Care and Use Committee at the University of Kufa, Iraq.

2.2. Experimental Design

Rats were randomly divided into four groups (n=7): sham group (negative control, similar surgical procedures to the control group without induction of IRI), control group (positive control, IRI group), vehicle group (similar to the control group but

preinjected with the vehicle which is distilled water), LPS-RS group (similar to the control group but preinjected with LPS-RS at a dose of 0.5 mg/kg body weight). The LPS-RS was purchased from Invivogen, USA. The compound was dissolved in endotoxin-free distilled water and injected intraperitoneally to the LPS-RS group 1 h before IRI induction.

2.3. Renal Ischemia/Reperfusion Injury Procedure

The IRI of the kidneys was induced bilaterally as previously described (9, 10). Briefly, the rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) (11). After that, an abdominal incision was made and both renal pedicles were clamped for 30 min using non-traumatic vascular clamps. About 1 ml of normal saline was administered into the abdominal area and the incision sites were covered with a moist piece of gauze to keep the animals in a well-hydrated status during the procedure (12). The reperfusion period started with removing the clamps and continued for 2 h. Later, the blood was collected by cardiac puncture and the kidneys were immediately collected after scarifying the animals (13).

2.4. Sample Collection

2.4.1. Serum Samples

Blood was collected by cardiac puncture and placed into gel tubes without anticoagulant and allowed to clot for 20 min. Centrifugation was done for 10 min at 3000 rpm to collect the serum samples (14). The collected serum samples were then used for the determination of urea, creatinine, and neutrophil gelatinase-associated lipocalin (NGAL) levels for the assessment of kidney function.

2.4.2. Tissue Samples

After collecting kidney samples, parts of the left kidneys were washed several times with cold phosphate-buffered saline to remove any residual blood clots or cells. After that, the tissues were homogenized (1:10 weight of tissue/volume of lysis buffer) in cold lysis buffer that contained PBS at pH 7.4 together with 1% of each of Triton-X 100 and protease inhibitor cocktail using a high-intensity liquid processor (15). Next, the homogenates were centrifuged at 4 °C and at

a speed of 4000 rpm according to the instructions of enzyme-linked immunosorbent assay (ELISA) kits. Later, the supernatants were collected and placed into different aliquots to be used for the determination of IL-1 β , IL-6, TNF- α , NGAL, and 8-isoprostane (as a marker of oxidative stress) levels using ELISA.

2.5. Statistical Analysis

Statistical analysis and figures were done in SPSS software (version 21). Data were analyzed by one-way analysis of variance followed by Tukey post-hoc test for multiple comparisons among different groups. It should be noted that a p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of LPS-RS on Serum Urea and Creatinine Levels

The serum urea and creatinine levels in the control and vehicle groups were significantly elevated in response to renal IRI, compared with the sham group. On the other hand, a similar trend was found in the LPS-RS group where the serum urea and creatinine levels were comparable to those of the control group and significantly higher than the sham group as shown in figures 1 and 2. These results indicated that LPS-RS pretreatment failed to ameliorate kidney function after IRI.

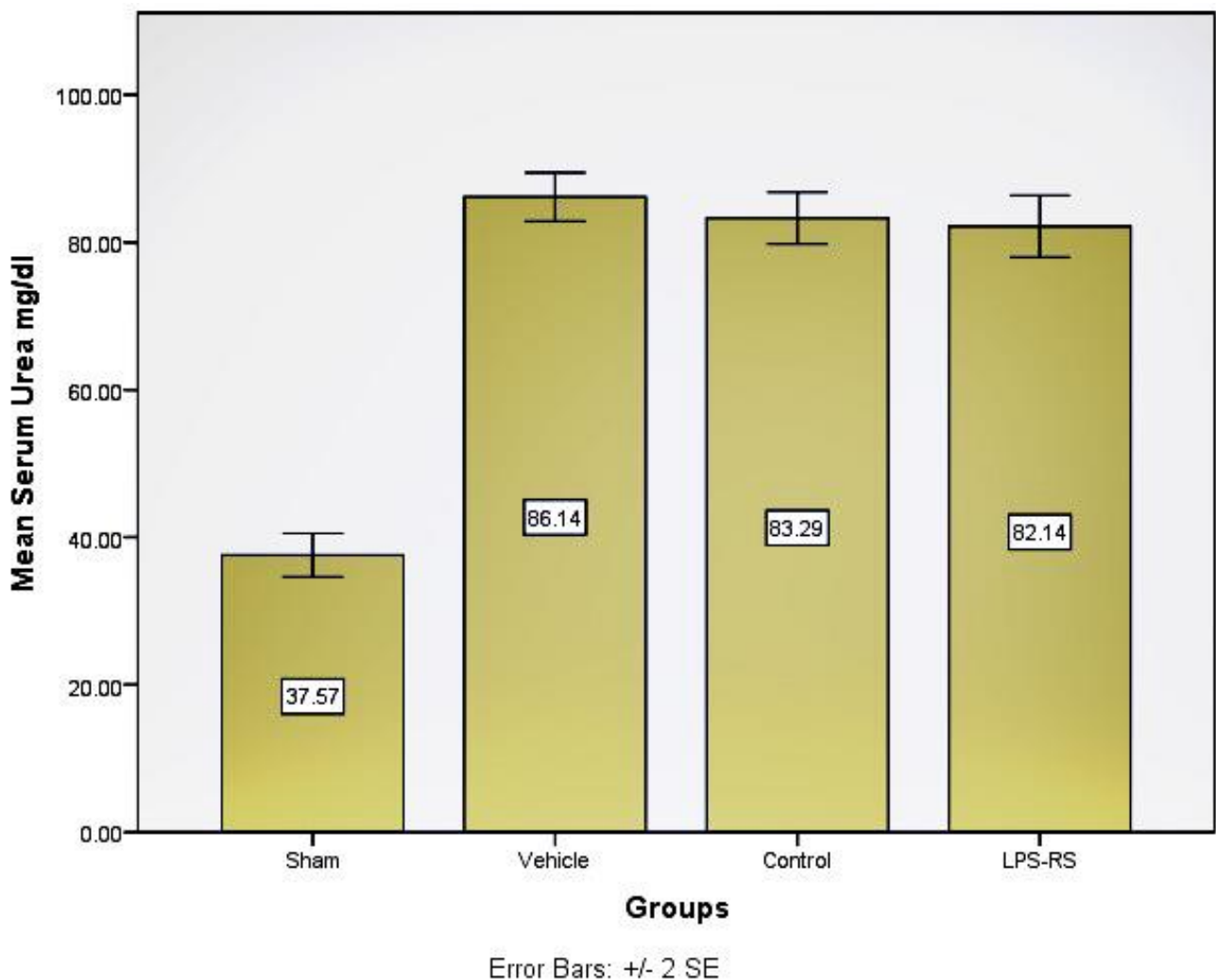


Figure 1. Variations in serum urea levels among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups (P<0.05).

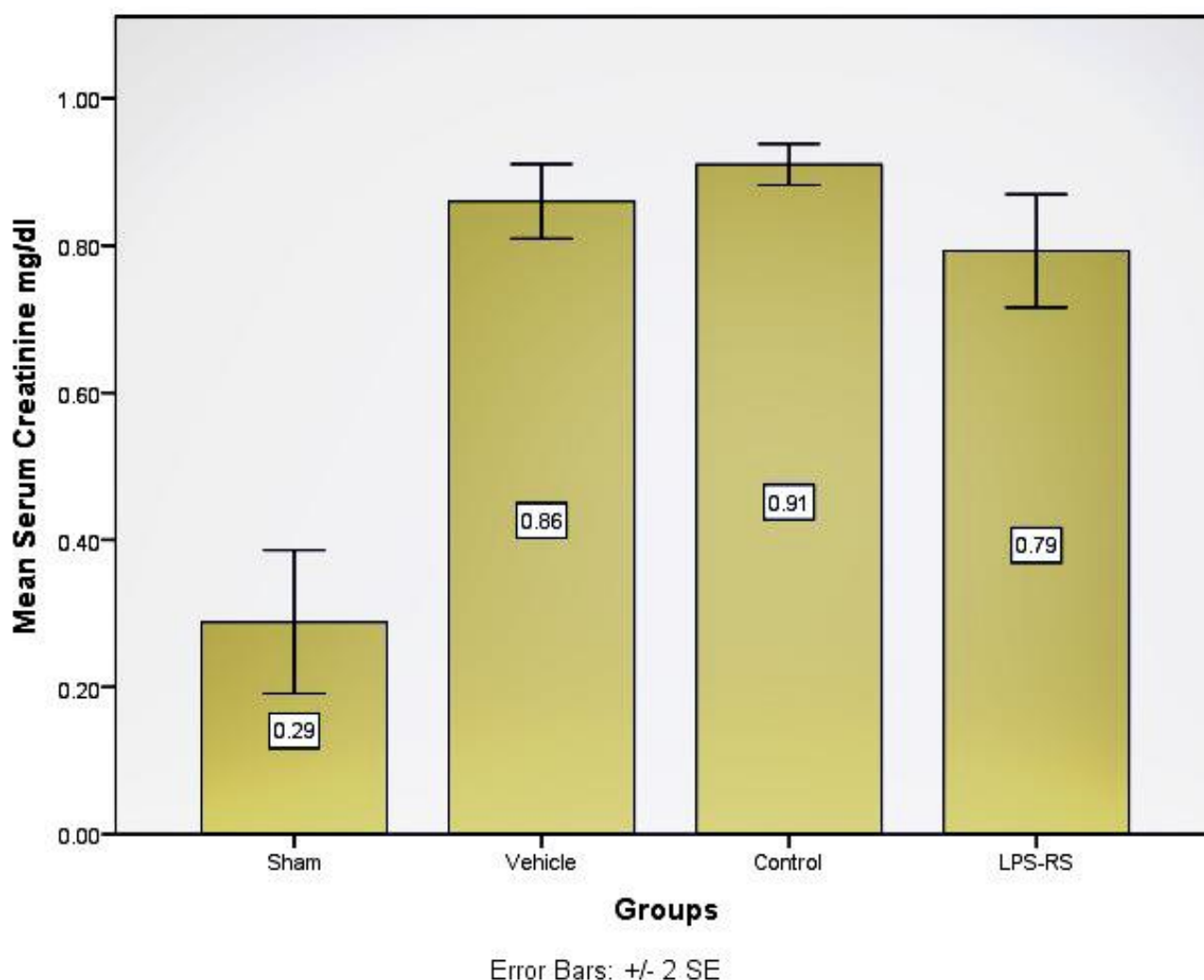


Figure 2. Variations in serum creatinine levels among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups (P<0.05).

3.2. Effect of Lipopolysaccharide Obtained from *Rhodobacter sphaeroides* on Serum Neutrophil Gelatinase-Associated Lipocalin Levels

As a marker of structural damage to the kidneys, NGAL was also investigated in the present study. The results revealed significantly elevated NGAL levels in the control and vehicle groups, compared to the sham group. Additionally, pretreatment with LPS-RS did not alter NGAL levels where the post-hoc analysis exhibited significantly higher NGAL levels, compared to the sham group with no significant difference in comparison with the control groups as shown in figure 3. These results along with the results of urea and

creatinine indicate that pretreatment with LPS-RS did not improve the renal dysfunction that occurred in response to the induced IRI.

3.3. Effect of Lipopolysaccharide Obtained from *Rhodobacter sphaeroides* on Renal Tissue Levels of IL-1 β

The alterations in the inflammatory mediator IL-1 β were also measured in this study. Significantly elevated renal tissue levels of IL-1 β were found in the control and vehicle groups, compared to the sham group. Furthermore, IL-1 β was significantly reduced in comparison with the control group but it was significantly higher than the sham group (Figure 4),

which could partly explain the persistent renal dysfunction seen after LPS-RS treatment.

3.4. Effect of LPS-RS on Renal Tissue Levels of IL-6

Another inflammatory mediator that was investigated in this study was IL-6. Tissue levels of IL-6 were

significantly higher in the control and vehicle groups in comparison with the sham group. On the other hand, IL-6 in the LPS-RS group was significantly higher than the sham and lower than the control group as shown in figure 5.

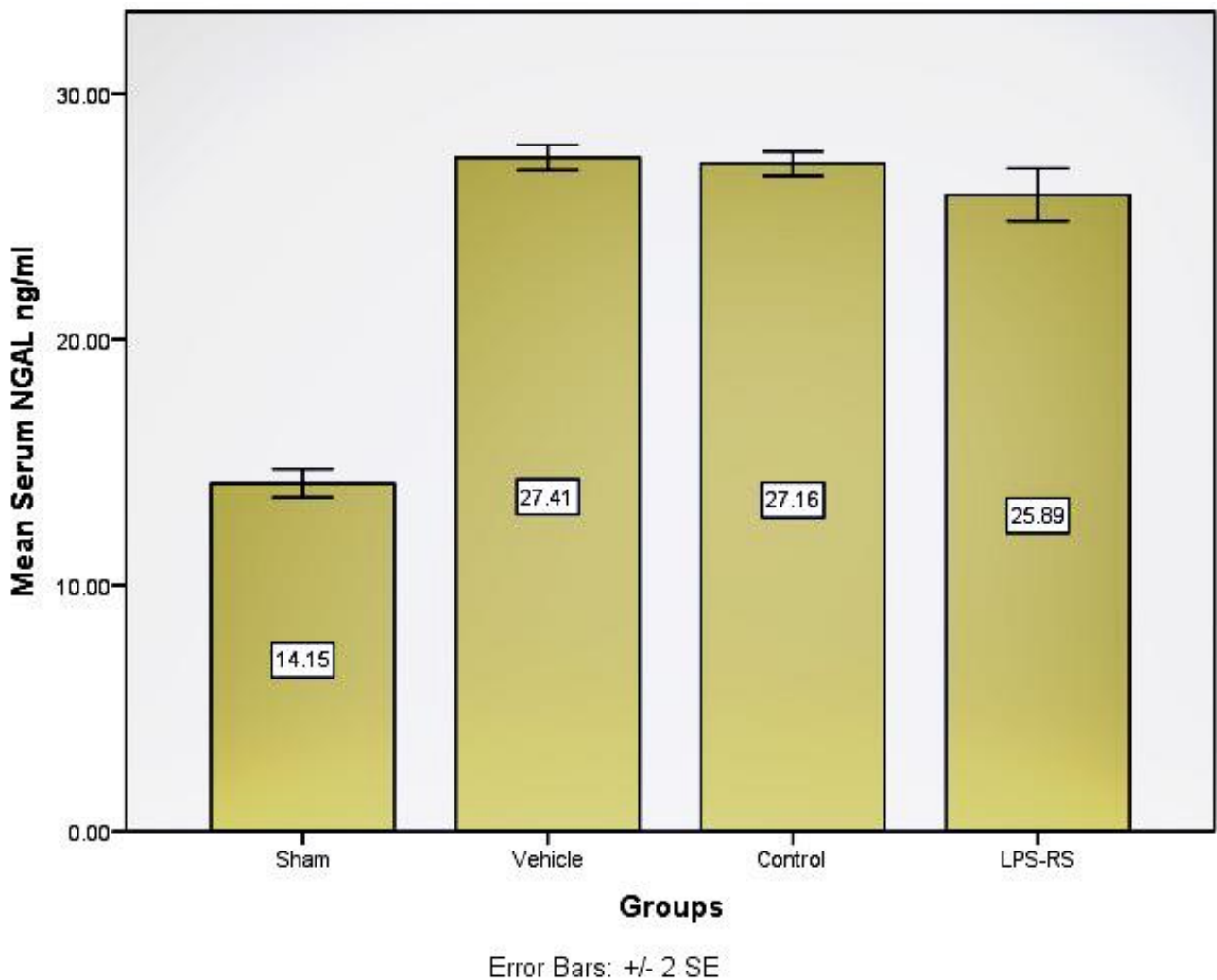


Figure 3. Variations in serum neutrophil gelatinase-associated lipocalin levels among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups (P<0.05).

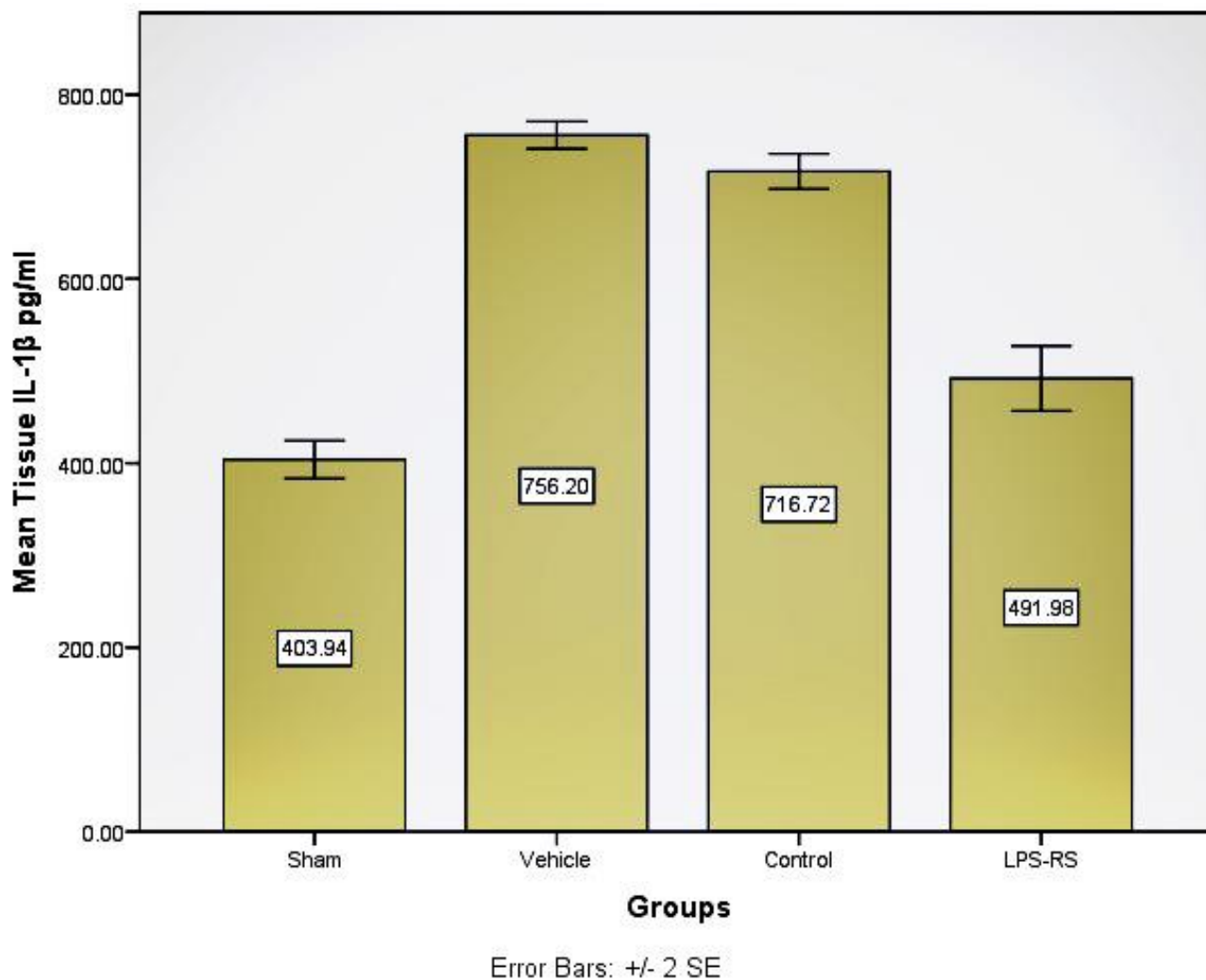


Figure 4. Variations in renal tissue IL-1 β levels among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups (P<0.05). LPS-RS group vs. control group (P<0.05).

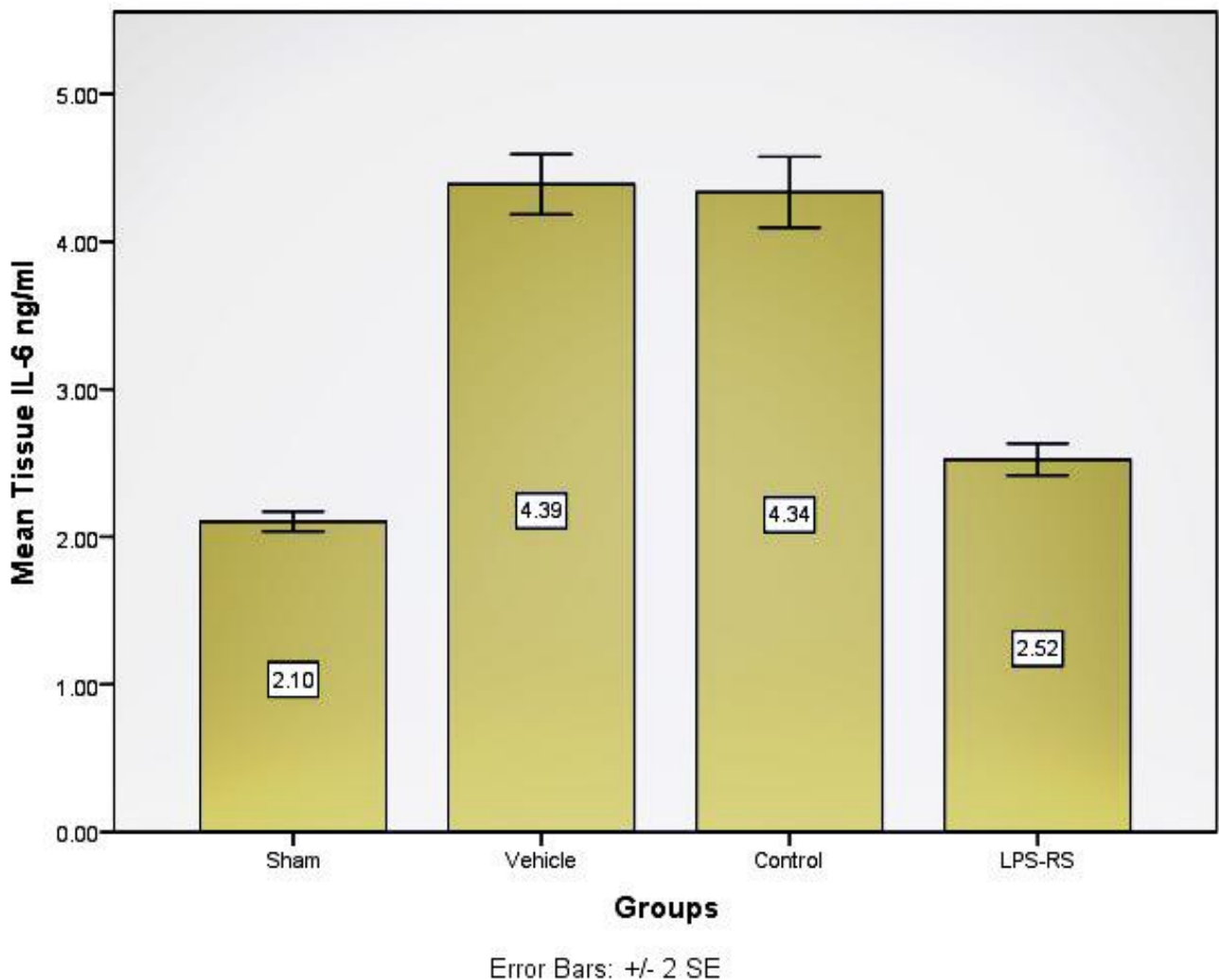


Figure 5. Variations in renal tissue IL-6 levels among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* (LPS-RS) groups ($P<0.05$). LPS-RS vs. control group ($P<0.05$).

3.5. Effect of Lipopolysaccharide Obtained from *Rhodobacter sphaeroides* on Renal Tissue Levels of TNF- α

Further evaluation of the inflammatory response was carried out by measuring TNF- α . The renal tissue levels of TNF- α were found to be significantly elevated after IRI in the control and vehicle groups, compared to the sham group. On the other hand, TNF- α levels were significantly higher in the LPS-RS group, compared with the sham group and significantly lower than the control group as shown in figure 6.

3.6. Effect of Lipopolysaccharide Obtained from *Rhodobacter sphaeroides* on Renal Tissue Levels of 8-Isoprostane

Oxidative stress is well known to play an important role in the pathogenesis of IRI. Therefore, the renal tissue levels of 8-isoprostane were investigated as a marker of oxidative stress. The results revealed a significant elevation in 8-isoprostane levels in the control and vehicle groups, compared to the sham group. Moreover, 8-isoprostane levels were significantly higher than the sham and lower than the control groups as shown in figure 7.

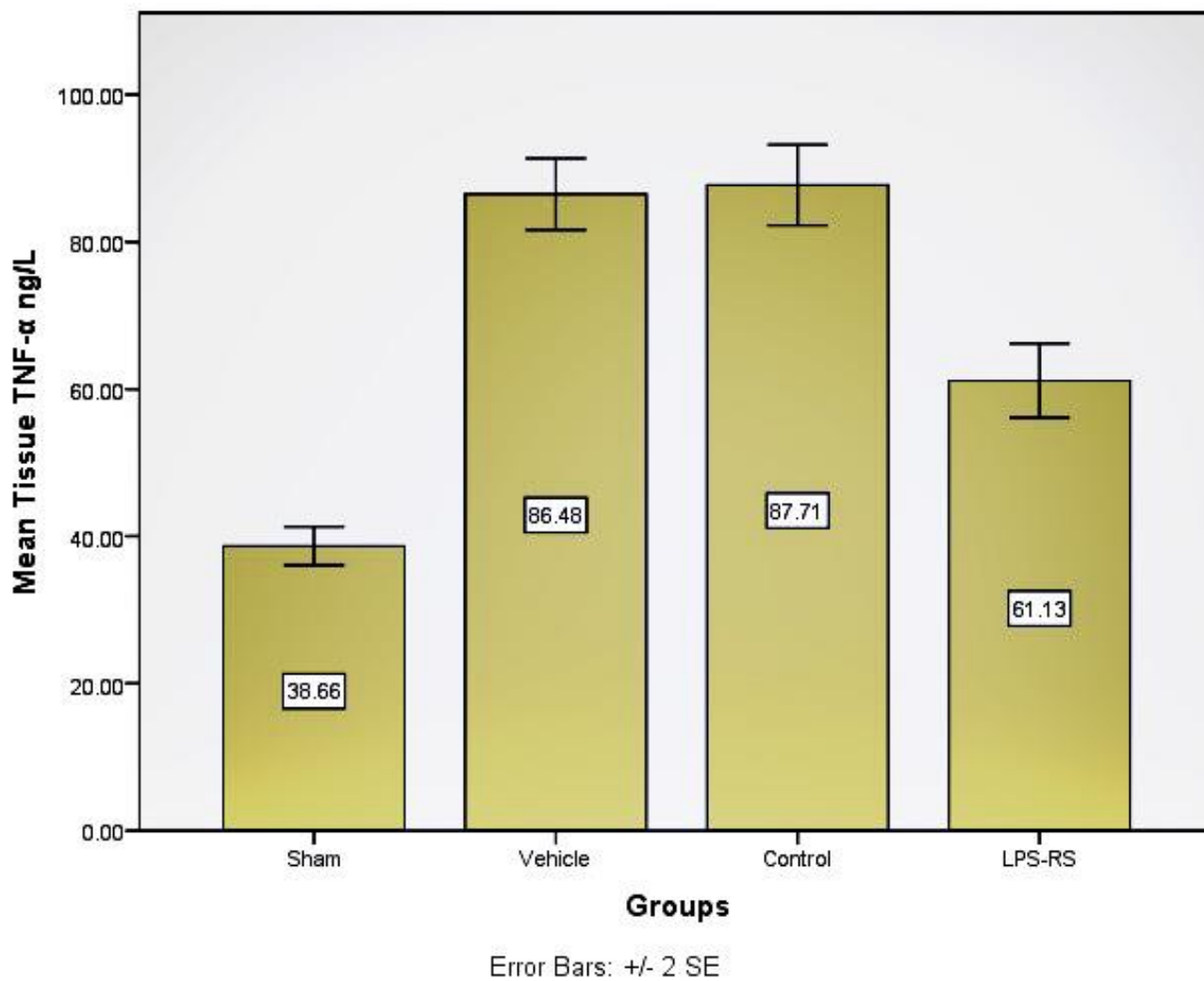


Figure 6. Variations in renal tissue levels of TNF- α among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups (P<0.05). LPS-RS vs. control group (P<0.05).

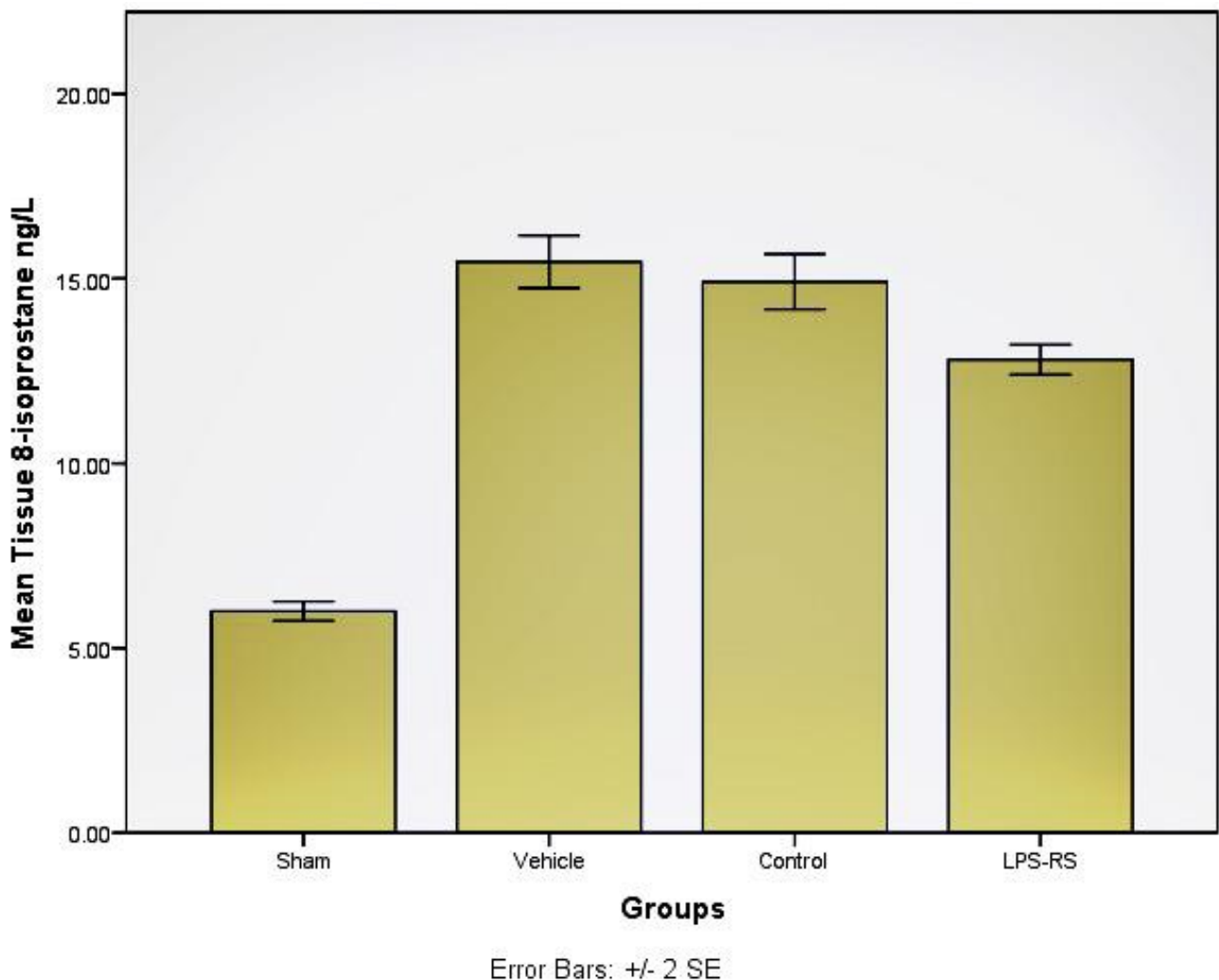


Figure 7. Variations in renal tissue levels of 8-isoprostane among the four experimental groups (n=7 in each group). Sham group vs. control, vehicle, and lipopolysaccharide obtained from *Rhodobacter sphaeroides* groups ($P < 0.05$). LPS-RS group vs. control group ($P < 0.05$).

4. Discussion

For the first time, in the present study, it was shown that pretreatment with LPS-RS offered no protection against renal IRI. After treatment with LPS-RS, there was persistent renal dysfunction manifested by the elevation of serum urea, creatinine, and NGAL levels. These effects of LPS-RS were found to be associated with elevations in the levels of the measured inflammatory cytokines (IL-1 β , IL-6, and TNF- α) along with the high levels of the oxidative stress biomarker 8-isoprostane in comparison with the sham normal group.

The animal model of bilateral renal IRI used in this study is a well-established *in vivo* animal model. Different times of ischemia and reperfusion have been chosen in previous studies (16-18). In the present study, it was decided to induce bilateral renal ischemia for 30 min as it was known from previous reports to cause significant acute kidney injury (9, 12). Moreover, severe renal damages have been previously reported to occur 2 h after reperfusion (10). Male rats were chosen for the study as they have been shown to be susceptible to renal IRI (19).

The LPS-RS is a lipopolysaccharide that has been isolated from the non-pathogenic bacterium *R. sphaeroides*. It is structurally different from the pathogenic lipopolysaccharides that are known to stimulate TLRs in that the lipid A of LPS-RS is a penta-acyl lipid unlike the hexa acylated lipids of the pathogenic ones (20, 21). This difference in the structure is the reason behind the ability of LPS-RS to antagonize TLRs. The LPS-RS has been tested previously and proposed as mixed antagonist of TLR2 and TLR4 in different pain models (8, 22). The timing of LPS-RS administration and the dose chosen in this study were based on previous studies (23, 24).

The results of the current study revealed significantly elevated serum levels of urea and creatinine in the control and vehicle groups. These results indicated the presence of renal dysfunction and are in line with those of a previous work that have shown urea and creatinine to be elevated in just 2 h after bilateral IRI in rats (25). Additionally, the serum NGAL levels, a more accurate and reliable marker of acute kidney injury also showed a similar pattern as urea and creatinine in accordance with previous studies (26, 27). Unexpectedly, none of the previously mentioned parameters were reduced after treatment with LPS-RS, indicating the persistence of renal dysfunction. These results were contradictory to those of previous studies that have shown inhibition of either TLR4 or both TLR2 and TLR4 to be effective in ameliorating the renal damages induced by IRI (17, 28). The lack of renoprotective effect after LPS-RS treatment may be due to other mechanisms that need to be explored; hence, further studies are needed.

The inflammatory response has been shown to play a pivotal role in the renal IRI and the central coordinator of this response is the TLRs, specifically TLR2 and TLR4 (4, 29). These receptors can ultimately activate the production of IL-1 β , IL-6, and TNF- α (6). Accordingly, all of these cytokines were found to be elevated in the control and vehicle groups, compared to the sham group, which were in line with previous studies (30, 31).

On the other hand, all of these parameters were significantly lower in the LPS-RS group, compared to the control group. Results of previous studies have shown nephroprotective effects following treatment with TLR4 inhibitors, an effect that was associated with a reduction in IL-1 β , IL-6, and TNF- α (32, 33). This does explain the observed reductions in the levels of these markers in the LPS-RS group; however, it does not explain why the renal dysfunction was not ameliorated following the treatment with LPS-RS. Furthermore, the levels of these parameters were higher than those in the sham group which could be the reason behind the persistent damage.

Oxidative stress has been shown to be a major mediator of the IRI of the kidneys by triggering cell death programs. Moreover, it has been shown that reactive oxygen species are produced during the ischemic phase with even larger amounts released from the mitochondria during the reperfusion (34). Furthermore, another important source of reactive oxygen species is the neutrophils that have been shown to be involved in IRI as early as 30 min after reperfusion (35). This could explain the significant elevations in the levels of 8-isoprostane in the control and vehicle groups, compared to the sham group. The 8-isoprostane, also called F2-isoprostane, was selected in this study as it is known as one of the most reliable markers of oxidative stress (36).

Alternatively, the renal tissue levels of 8-isoprostane in the LPS-RS groups were significantly lower than in the control group. Previous studies with TLR4 antagonists have shown a reduction in oxidative stress following the use of those compounds and it has been concluded that such effect can partly mediate their renoprotective effect (17, 37). However, in the present study, the renal dysfunction continued since the levels of 8-isoprostane were almost two times higher than the sham group and this could partly mediate the ongoing renal dysfunction seen in the LPS-RS group.

In conclusion, the results of the present study showed for the first time that LPS-RS at the tested dose failed

to offer a significant protective effect against the renal dysfunction induced by renal IRI. This effect was accompanied by reduction, but not normalization, in the levels of the measured inflammatory and oxidative stress markers investigated in the current work. The results of the present study should be carefully interpreted as the compound might be effective at different doses and under different medical conditions. Therefore, further studies are needed to test different doses of LPS-RS to explain the reason for the lack of the renoprotective effect.

Authors' Contribution

Study concept and design: M. A.

Acquisition of data: A. R. A. R.

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

Drafting of the manuscript: M. A.

Critical revision of the manuscript for important intellectual content: N. R. H.

Statistical analysis: M. A.

Administrative, technical, and material support: A. R. A. R.

Ethics

This study was ethically approved by the Institutional Animal Care and Use Committee at the University of Kufa, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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