Pak Heart J 2023:56(02)
ISSN:0048-2706 E-ISSN:2227-9199

MAS Receptor Agonist (AVE0991) Attenuate Renal Ischemia-Reperfusion Injury A Mouse Model

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Abstract:
Renal ischemia/reperfusion injury (I/R) is a common cause of acute kidney injury (AKI) in patients, and renal blood stream obstruction is inevitable after renal transplantation. In the present study, we investigated the renal effects of acute administration of the Mas receptor agonist, AVE0991, in a murine model of AKI caused by reperfusion of ischemic kidneys. Mice were divided into four groups: control group, sham group, control vehicle group, and treatment of mice with AVE09. Histopathological analysis revealed an increase in tissue damage after IRI, as shown by the presence of vacuolization, dilated renal tubules, and glomerular alterations, as well as a dilatation of the Bowman's capsule and a lack of brush boundaries. Hematoxylin and eosin (H&E) staining revealed a decrease in the serum levels of interleukin-10 (IL-10), IL-6, and tumor necrosis factor-alpha (TNF-a). Treatment of mice, however, significantly improved renal injury (P 0.05) as compared to the control group and the overall severity score mean of this group revealed moderate renal impairment. The renoprotective effects of AVE 0991, when taken prior to renal I/R damage, were highly comparable to those induced by the AT1 receptor antagonist Losartan.
Keyword: MAS Receptor Agonist; (AVE0991); Renal Ischemia; Reperfusion Injury; Mouse Model

Introduction

1.1 Renal Ischemia/Reperfusion Injury

Renal I/R is an inflammatory process that results in acute kidney damage (AKI). AKI is a clinical condition characterized by a quick (hours to days) decrease in renal capacity and the accumulation of nitrogen digestive system products in the plasma, such as creatinine and urea. Other fundamental clinical indicators include decreased pee production (oliguria), aggregation of metabolic acids, and increased potassium and phosphate concentrations(Makris and Spanou, 2016).

AKI may occur not just after a kidney transplant, when I/R is unavoidable, but also as a result of impaired renal perfusion, such as after surgery or infection. For the kidney, IR is also caused by heart failure (systemic hypoperfusion), shock, surgical interventions causing local renal hypoperfusion, such as aortic cross-clipping, partial nephrectomy, and transplantation. Renal ischemia reperfusion injury (IRI) is a common cause of acute kidney injury (AKI) in patients, and renal blood stream obstruction is inevitable after renal transplantation. (Chatauret *et al.*, 2014)**.**

APT (adenine triphosphate) depletion, increased intracellular calcium, and activation of membrane phospholipids proteases are all effects of ischemia-reperfusion injury (IRI), which occurs when damaged tissue produces an excessive amount of reactive oxygen species (SOS)(Malek and Maleki, 2018).

Oxidative stress is the key mediator of ischemia-reperfusion injury due to the inability of the innate antioxidant defense system to buffer the large burst of free radicals, which ultimately result in membrane lipid peroxidation(Rovcanin *et al.*, 2016).

With endothelial activation, leukocyte recruitment, elevation of cytokines and chemokines, and subsequent ischemia and reperfusion, inflammation plays a significant role in the pathophysiology of ischemia reperfusion and contributes to cellular dysfunction(L. Wang *et al.*, 2018).

TLRs are expressed in leukocytes and renal tubular epithelial cells. TLRs activate innate immune responses by recognizing foreign microbial ligands (pathogen-associated molecular patterns [PAMPs])(Takeda and Akira, 2005).

TLR4 and its endogenous ligands were upregulated after kidney ischemia, and the TLR4/MyD88 pathway was necessary for kidney IRI formation. Kidney failure and histopathological damage were prevented in TLR4- or MyD88-deficient mice. We found that TLR4 signaling in renal parenchymal cells caused higher kidney damage than TLR4 expression on leukocytes in IRI(Wu *et al.*, 2007).

Materials and methods

1.1 Preparation of Animals

Twenty mature male mice weighing between 25 and 30 g were collected from the animal house at the Kufa University School of Science. have unlimited access to a regular mouse diet and water. There is an Ethical Committee at Kufa University that reviews and approves every research before it is conducted.

1.2 Design of the Study

The mice were divided into the following 4 groups (n = 5)

Group 1: Sham group: Under anesthesia, mice had a median laparotomy without clamping, and samples of kidney tissue and blood were taken.

Group 2: Control group: Anesthetized mice had a median laparotomy, then their kidneys were ischemic for 30 minutes on both sides, and then their kidneys and blood
were taken 2 hours after the kidneys had been reperfused. (Yasmeen A. Hussien *et al.*, 2020).

Group 3: Control vehicle group: Mice received DMSO vehicle for AVE0991 (Mi *et al.*, 2021)and compound 21(Schwengel *et al.*, 2016) intraperitoneal injection at 30 minutes before ischemia.

Group 4:AVE0991 treated group: Mice underwent median laparotomy under anesthesia after the administration of AVE0991 intraperitoneal injection of 9mg/kg (Barroso *et al.*, 2012) , 1h before the induction of ischemia followed by 30min bilateral renal ischemia, Renal tissues, and blood samples were collected after 2 h of reperfusion.

1.3 Induction of Ischemia/Reperfusion Injury as a Model of Acute Kidney Injury in mice

Mice get AEV0991 intraperitoneally 1 hour before surgery (treatment groups). Clamping both renal pedicles causes ischemia. Ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally anesthetize each animal. A midline abdominal incision clamps the two renal pedicles using non-traumatic vascular clamps (excluding sham group) (Barroso et al., 2012). To hydrate animals, 1 ml of 37°C warm saline was injected into the peritoneal cavity (0.5 ml when the clip was inserted and withdrawn). To maintain body temperatures, the mice are placed on a 38°C heating pad until they wake up. After reperfusion, animals will be sacrificed by heart puncture under ketamine and xylazine anesthesia to collect blood and kidney samples for analysis. After harvesting the kidneys, one is deep-frozen for molecular investigation while the other is fixed with 10% formalin and embedded in paraffin for histological examination.

1.4 Collection of Samples

At the conclusion of the reperfusion period, the mice were weighed on an electronic scale before being given an intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg) (Barroso et al., 2012). Prior to the exams, around 2 ml of blood were taken straight from the heart by cardiac puncture, and the kidneys were emptied.

1.4.1 Preparation of Serum Samples

The 2 ml of collected blood was placed in a tube without anticoagulant and allowed to sit at room temperature for 30 minutes before being used to obtain serum through centrifugation at 3000 rpm for 10 minutes. The serum was then used to estimate the levels of urea, creatinine, TNF alpha, IL-6, and interleukin - 10.

1.4.2 Tissue Preparation for MPO, F2-Isoprostane and TLR4 Measurement.

Kidneys were washed in cold saline to eliminate red blood cells and clots, and then homogenized using a high intensity ultrasonic liquid processor in a solution of 1:10 (w/v) 0.1 M (PBS) phosphate buffered saline (pH 7.4) containing 1% Triton-100 and protease inhibitor cocktail (AL-Sheibani et al., 2014).

Supernatants were collected after centrifuging the 10% homogenates at 10,000 rpm for 10 minutes at 4°C in order to measure MPO, 8-isoprostane, and TLR4 (Sabuhi et al., 2011).

1.4.3 Tissue Sampling for Histopathological Analysis and Damage Scoring

Hematoxylin and eosin (H&E) staining was performed after the kidney was fixed in 10% formalin and processed using standard histological techniques, and paraffin sections 5 m thick were cut from these sections. (Bancroft and Gamble, 2008).
Histological changes in the cortex and in the external stripe of the external medulla (OSOM) were assessed by quantitative estimations of tissue harm by a blinded observer. Tubular harm was characterized as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast development, and desquamation. histological sections from all groups were examined and scored according to the protocol of Jiang (Jiang *et al.*, 2010).The degree of kidney damage was estimated at ×200 magnification, using five randomly selected fields for each animal, by the following criteria: 0, no abnormality; 1, mild damage ; 2, moderate damage ; 3, severe damage ; and 4, highly severe damage

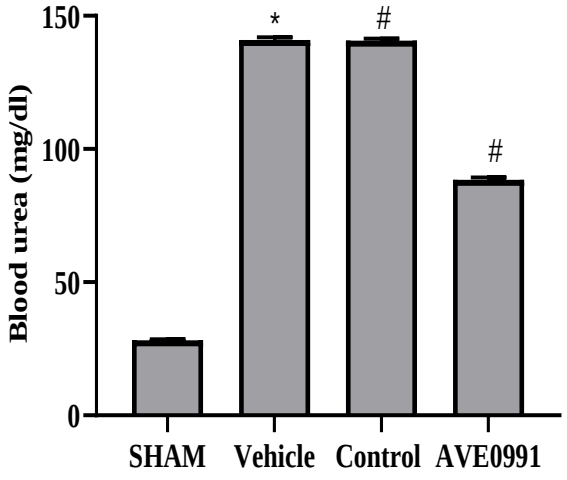
1.5 Statistical Analysis

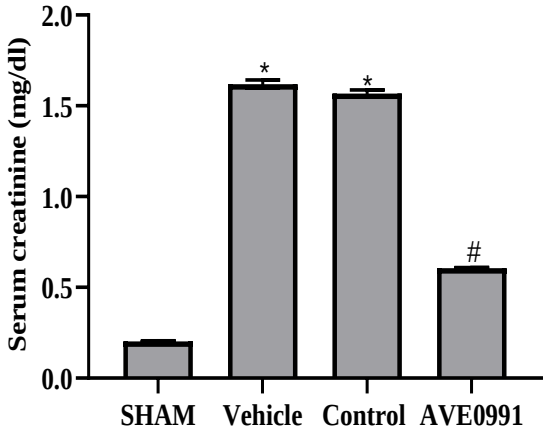
Data are expressed as mean ± standard error of mean (SEM) and the number of experiments (n) is mentioned in the legend of each figure. In addition, statistical analysis of data is explained in the legends. Graphpad prism version 8.1 (Graphpad software Inc, San Diego, USA) was used in the analysis of data and generation of significance. To analyse our data, the normality of distribution was tested using Shapiro–Wilk test followed by examining the difference between controls and treatments. One Way ANOVA with Bonferroni multiple comparison test was used to examine the difference between control and treated groups. Those data with p value<0.05 is considered statistically significant.

Results

1.1 Effects on Kidney Function Parameters (Serum Urea and Creatinine)

Serum level of Urea and Creatinine was significantly ($p < 0.05$) increased in (control) group as compared with the sham group. There was an insignificant difference between the control vehicle and control group. Serum level of Urea and Creatinine of AVE0991 treated group was significantly ($p<0.05$) lower than that of the control vehicle group. Variations in Serum level of Urea and Creatinine are summarized in figures (1 and 2)





Effect on serum IL-10

Ischemia/reperfusion injury resulted in significantly ($p>0.5$) lower serum levels of IL-10 in control when compared to SHAM. However, treatment with AVE0991 significantly improved the serum levels of IL-10 when compared to control (figure 3)

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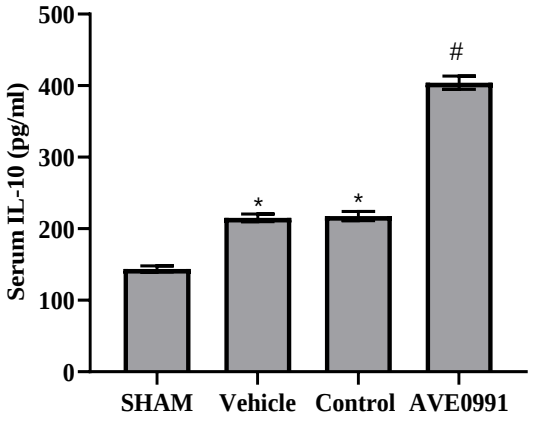
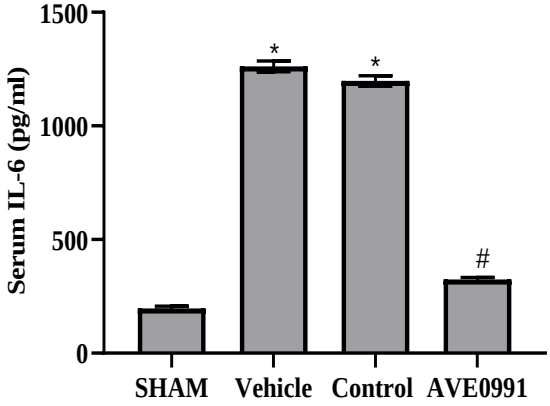


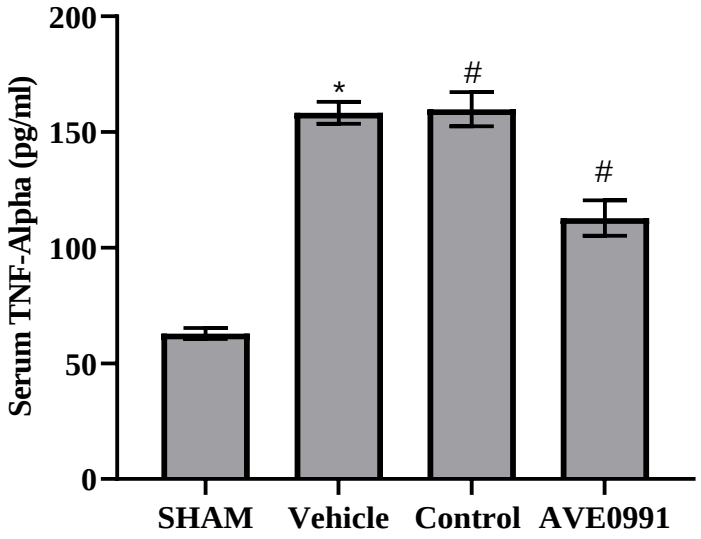
1.1 Effect on serum IL-6

After induction of ischemia/reperfusion injury, there was significantly (p>0.5) higher serum levels of IL-6 in control when compared to SHAM. Treatment with AVE0991, however, significantly resulted in lower serum levels of IL-6 when compared to control (figure 4).



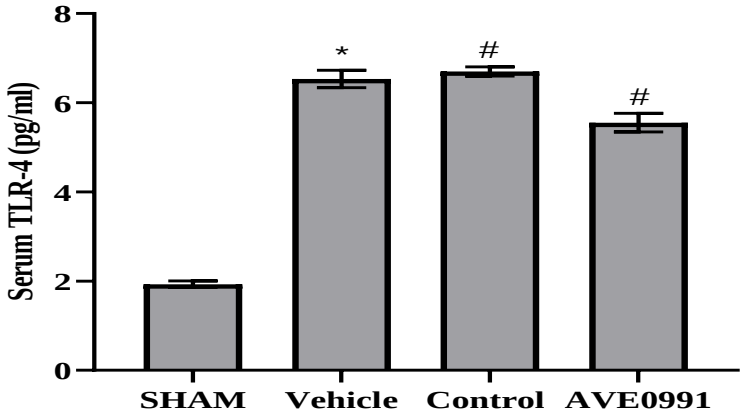
1.1 Effect on serum TNF-α

Significantly (p>0.5) higher serum levels of TNF-α were shown in ischemia/reperfusion injury group (control) when compared to SHAM. Treatment with AVE0991 ,however, significantly resulted in lower serum levels of TNF-α when compared to control (figure 5).



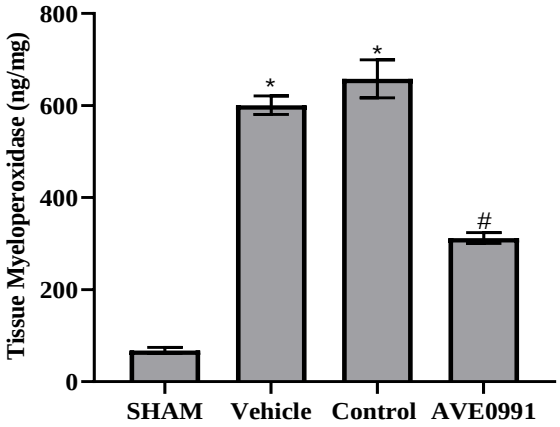
1.2 Effect on renal tissue TLR-4

In animals subjected to I/R injury(control), there were significantly (p>0.5) higher tissue levels of TLR-4 receptor when compared to SHAM. Treatment with AVE0991 significantly resulted in lower tissue levels of TLR-4 receptor when compared to control (figure 6).



1.1 Effect on renal tissue myeloperoxidase

I/R injury (control) resulted in significantly (p>0.5) higher tissue levels of myeloperoxidase when compared to SHAM. Treatment with AVE0991 significantly resulted in lower tissue levels of myeloperoxidase when compared to control (figure 8).



1.1 Histopathological Finding

Acute kidney injury was assessed in the renal mice of the four experimental groups at the end of the study and the results are as follows:

1.1.1 Sham group

A cross-section of the sham renal mice showed normal renal structure. All mice in this group showed normal renal tissue as shown in table (3.1)& Figure (3.9).

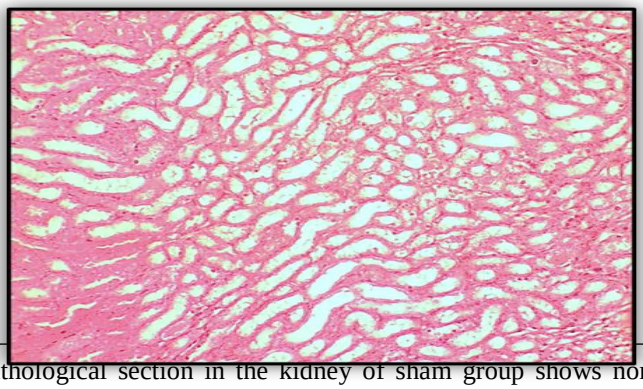


Figure 3-9: The histopathological section in the kidney of sham group shows normal renal tubular . The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnifier scale.

Control group

There was a statistically significant difference between the control group and sham group ($P < 0.05$) and the total severity scores of the control group showed that 80% of this group had a severe renal injury, 20% had a highly severe renal injury as shown in table (3.1) and figure (3.10)

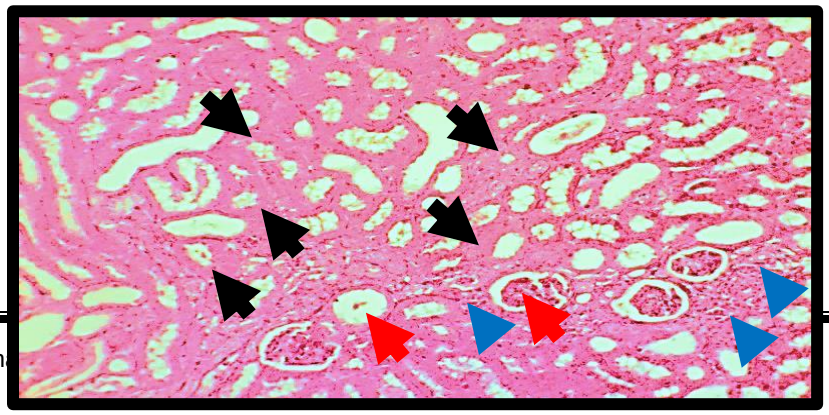


Figure 3-10. The histopathological section in the kidney of mice in control group shows mild atrophied lesion in the glomerular tufts (Black arrows) and clearly increasing in the Bowman's space (Red arrows), in addition, there is a mild renal tubules hypertrophy (Blue arrows). The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnifier scale.

Control vehicle group (DMSO group)

There was the statistically insignificant difference between control vehicle group and control group (P>0.05) and the total severity scores of the control vehicle group showed that 60% of the group had a severe renal injury, 40% had a highly severe renal injury, As shown in table (3.1)and figure (3.11)

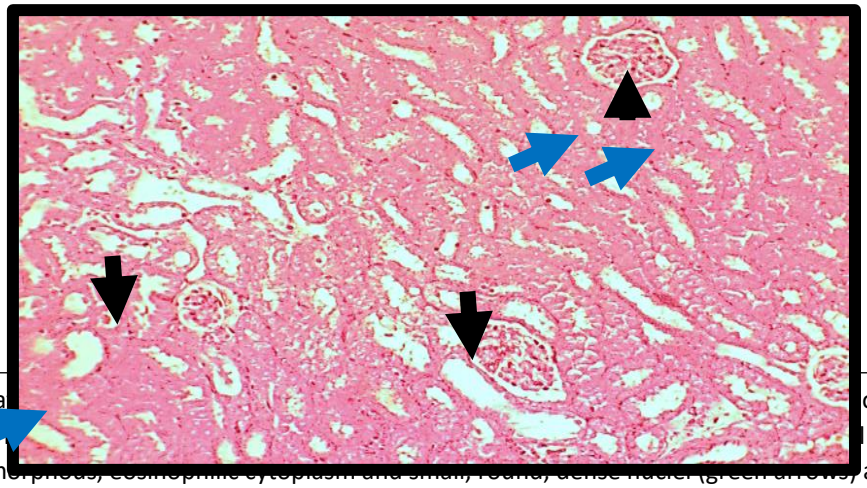


Figure 3-11. The histopa omerular texture (Black arrow) and sev cells explain an increased amount of am are associated with chronic progressive nephropathy. The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnifier scale.

AVE0991 treated group

Treatment of mice with **AVE0991** improved renal injury significantly (*P* <0.05) as compared with the control vehicle group and the total severity score mean of this group showed that 20% of the group had a mild renal injury and 80% had moderate renal injury, As shown in table **(**3.1)and figure (3.12)

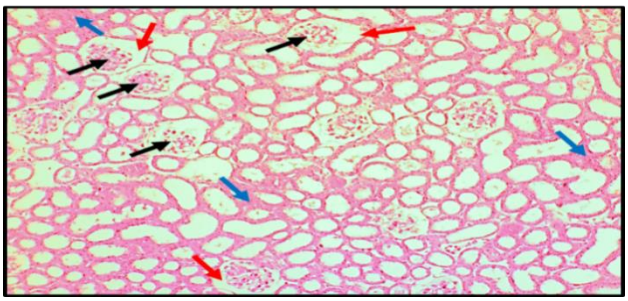


Figure 3-12. The histopathological section of kidney in mice in group that treated with (AVE , dose 9mg/kg) shows normal texture of renal tissue (renal proximal tubules, Black arrows and renal glomeruli, Red arrow) with mild Renal tubule - hypertrophy in mice (Blue arrows). The tissue is stained by H&E stain and the section is captured using light microscope and digital camera at 10X magnifier scale.

Table 3-1 The differences in the histopathological grading of abnormal renal changes in the experimental groups.

Histological grading	Sham		Control		DMSO		AVE	
	N	%	N	%	N	%	N	%
No abnormality (0)	5	100	0	0	0	0	1	20
Mild (1)	0	0	0	0	0	0	3	60
Moderate (2)	0	0	1	20	2	40	1	20
Severe (3)	0	0	4	80	3	60	0	0
Total	5	100	5	100	5	100	5	100
Score	No Abnormality		Sever		Sever		Moderate	

Discussion

Ischemia/reperfusion injury is a term that is used to describe the experimentally and clinically common finding that tissue ischemia with insufficient oxygen followed through successful reperfusion initiates a massive and complex array of inflammatory responses that can irritate immediate damage as well as induce impairment of remote organ function by means of mechanisms that have been contained within the tissue. This finding is known as the ischemic-reperfusion injury cascade.(Hadi *et al.*, 2017).

There were previously studies shown that RAS activation has an important role in the development of ischemic AKI,

1.3 Effect of Renal I/R on renal function parameters (urea and creatinine)

1.2

mediating inflammatory and profibrotic actions through AT1R(Molinas *et al.*, 2009).

Recently, studies have also demonstrated an important role of the counter regulatory RAS axis, ACE2/Ang- (1–7)/Mas receptor, in several renal disorders, including AKI(Liu *et al.*, 2022)

In the present study, we investigated the renal effects of acute administration of the Mas receptor agonist, , in a murine model of AKI caused by reperfusion of ischemic kidneys.

In this study, I/R caused a higher level of urea and creatinine in control and control vehicle group as compare with sham group. This result are in an agreement with those reported by Abd-Jabbar et al (Abd-Jabbar *et al.*, 2019)revealed that I/R caused a critical rise in serum levels of creatinine after 30-min ischemia 2-h reperfusion.

Hussien et al .(Yasmeen A Hussien *et al.*, 2020) found that the untreated IRI showed a higher level of urea and creatinine in control group as compared with the sham group .

Jia et al., 2016 (Jia *et al.*, 2016)showed that the ischemia reperfusion group showed significantly higher concentration of creatinine and urea after 45 minute ischemia ,24 hour reperfusion in a rat model .

1.4 Effect of AVE0991 on renal function parameters (urea and creatinine)

In this study, AVE0991 significantly lower the levels of urea and creatinine when compared with that of control group demonstrating preservation of renal function . This results is in consistence with (Barroso *et al.*, 2012)their results found significant decrease in the levels of urea and creatinine in AVE0991 treatment group as compare with the ischemia reperfusion group when the mice subjected to 30 mint ischemia and 24 hours reperfusion .

1.5 Effect of RIRI on pro-inflammatory cytokines (TNF-α and IL-6)

This study found a significant increase ($P<0.05$) in inflammatory mediators (TNF-α and IL-6) in the control and control vehicle groups when compared to the sham group. Yuan, Wang and Zhang show that the pro inflammatory cytokines (TNF-α and IL-6) serum level elevated during ischemia reperfusion injury in murine model (Yuan, Wang and Zhang, 2020) .Pro-inflammatory cytokines and cytokines such as interleukin 6 (IL6) and TNFα play a major role in renal dysfunction of IRI(Patel *et al.*, 2005). . In other study, TNF-α, IL-1β and IL-6 significantly increased in I/R group (Unsal *et al.*, 2021). All these studies are in consistence with our study.

This results suggest the role of the overproduction ROS and proinflammatory cytokines in intense cellular and tissue injury by inducing oxidative damage of inflammatory cell infiltration and biological macromolecules (Patschan, Patschan and Müller, 2012).

1.6 Effect of AVE0991 on pro-inflammatory cytokines (TNF-α and IL-6)

Our study found that AVE0991 may have anti-inflammatory properties as significantly reduce level of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)this study is in a consistence with Qi et al that anti-inflammatory effect of Ang.(1-7) reduce the pro-inflammatory cytokines and elevate the level of anti-inflammatory cytokine IL-10(Qi *et al.*, 2011).Another study also agreed with out study that found cytokines such as TNF-α and IL-6 were also significantly attenuated in AVE0991-treated mice(Skiba *et al.*, 2017)

1.7 The effect of RIRI on serum IL-10

A baseline level of IL-10 expression was found in normal renal tissue. After the ischemic event, there was a rise in serum IL-10 levels. In our study we found that the serum IL-10 was raised 2 hour after RIRI .This study is in consistence with previous study by Sakai et al (Sakai *et al.*, 2019)

In contrast another study show lower of serum IL-10 level after RIRI (Collino *et al.*, 2013)

1.8 The effect of AVE0991 on serum IL-10

However, treatment with AVE0991 elevate the level of IL-10 when compared to control .Although increased production of the anti-inflammatory cytokine interleukin-10 is one indication that Ang-1-7, which has anti-inflammatory characteristics, is present(Qi *et al.*, 2011) .

To the best of our knowledge there is no research has measured the effect of AVE0991 on IL-10 serum level

1.9 The effect of RIRI on renal tissue TLR-4

In the current study, there was a substantial rise in tissue TLR4 levels in the control and control vehicle groups as compared to the sham group. Furthermore, the current research found that the TLR4 signaling pathway is critical in renal IRI-associated inflammation and apoptosis. This conclusion is consistent with the findings of (Vallés *et al.*, 2023).
Pulskens et al (Pulskens *et al.*, 2008) TLR4 performs a proinflammatory function, as shown by lower levels of invading granulocytes and chemokine in TLR4 -/- mice kidneys compared to wild type mice. TLR2 and TLR4 are upregulated following ischemia or nephrotoxic drugs playing a pro-inflammatory role (Cucchiari, Podestà and Ponticelli, 2016)another study also in consistence with our study. (Wu *et al.*, 2007)

1.10 The effect of AVE0991 on renal tissue TLR-4

Treatment with AVE0991 significantly resulted in lower tissue levels of TLR-4 when compared to control this was shown in our study also LI et al (LI *et al.*, 2018) propose that Ang-(1-7) protects MACO rats against brain ischemia-reperfusion damage, and its mechanism may be connected to the suppression of the TLR4/NF-B pathway. Another study show that Ang 1-7 may be considered an anti-inflammatory factor that serves to downregulate the TLR4/NF-κB signaling pathway during the pathogenic progression of acute pancreatitis (Y. Wang *et al.*, 2018).

1.11 The effect of RIRI on renal tissue myeloperoxidase

I/R injury (control) resulted in significantly higher tissue levels of myeloperoxidase when compared to SHAM. This result in a consistence with another study which reveal that renal I/R caused an elevation in tissue MPO activity indicating the presence of enhanced PMN recruitment in the inflamed tissue(Tuğtepe *et al.*, 2007). (Altintas *et al.*, 2013) demonstrate that the renal ischemia reperfusion injury elevate renal tissue MPO level after ischemia. Also more recent study by (Nezamoleslami *et al.*, 2020) As shown in this study the IR group the kidney tissue level of MPO after I/R was significantly raised compared to the sham group .

1.12 The effect of AVE0991 on renal tissue myeloperoxidase(MPO)

This study shows that the treatment with AVE0991 significantly resulting in lower tissue level of MPO when compared to the control. To the best of our knowledge there are no studies investigate the effect of AVE0991 or angiotensin (1-7) on the renal tissue myeloperoxidase but there are studies of angiotensin (1-7) on the lung myeloperoxidases, one of which was shown that Ang-(1–7)/Mas regulates MPO activity and the production of proinflammatory components. The studies demonstrated that Ang-(1–7)/Mas protected against acute lung damage by acting directly on neutrophils(Wang *et al.*, 2022).

1.13 Effect of Renal I/R injury on oxidative stress marker (8- isoprostane)

In this present investigation, it was discovered that the control group had much higher levels of 8-isoprostane than the sham group. Elevated oxidative stress and reactive oxygen species generation are important in initiating and maintaining the inflammatory response. This study is in consistence with (Colli *et al.*, 2013) where the findings revealed a trend indicating that 8-isoprostane levels were greater after renal clamping compared to 8-isoprostane levels before renal clamping. Another study also show that the 8-isoprostane level as a real-time biomarker for renal ischemia dramatically rose in rats when the renal hilum was clamped(Alirezaei *et al.*, 2017). (Zaman and Banday, 2022) show that as compared to sham rats, I/R elevated blood pressure, plasma creatinine, urine 8-isoprostane, renal infiltration of pro- and anti-inflammatory macrophages, and decreased glomerular filtration rate in both adult and elderly rats.

1.14 Effect of AVE9001 on oxidative stress marker (8-isoprostane)

This study shows that the treatment with AVE0991 significantly resulting in lower tissue level of 8-Isoprostane when compared to the control. To the best of our knowledge there are no studies investigate the effect of AVE0991 or angiotensin (1-7) on the renal tissue 8-isoprostane but (Potthoff *et al.*, 2014) shows that Ang-(1–7) causes a decrease in oxidative stress, which in turn leads to a reduction in the activity of the p38 mitogen-activated protein kinase. These actions are mediated via the Mas-receptor.
(Xu *et al.*, 2008) show a decrease in superoxide dismutase and catalase activity in Mas-deficient mice from two separate genetic backgrounds, showing weakened antioxidant characteristics in these animals.

1.15 Effect of renal IR injury on Renal parenchyma

Histological analysis revealed an increase in tissue damage after IRI, as shown by the presence of vacuolization, dilated renal tubules, and glomerular alterations, as well as a dilatation of the Bowman's capsule and a lack of brush boundaries. These changes had been in agreement with some other studies (Collino *et al.*, 2013) , (Dey, 2018) and (Kang *et al.*, 2014) that showed the same histopathological changes .

1.16 Effect of AVE0991 on Renal Parenchyma

The treatment of mice with AVE0991 significantly reduced renal damage as compared to the control vehicle group, and the overall severity score mean of this group revealed moderate renal impairment. The current research demonstrated that AVE0991, when taken prior to renal I/R damage, prevents kidney harm through histopathological parameters. The current study's findings are consistent with those revealed by prior research (Barroso *et al.*, 2012) show that the renal tissue of AVE-treated animals had a significant decrease in the indexes of renal injury at both glomerular and tubular sites when compared to vehicle group also (Silveira *et al.*, 2013) reveal that In a mouse model of ADR-induced nephropathy, therapy with AVE 0991, an orally active Mas receptor agonist, dramatically improved renal function indices, decreased urinary protein loss, and mitigated histological abnormalities.

The renoprotective effects of AVE 0991 were highly comparable to those induced by the AT1 receptor antagonist Losartan.

Giani *et* al shows that renal fibrosis is improved and inflammatory cytokine expression is decreased in kidneys of hypertensive rats when ANG-(1-7) is administered also reveal that ANG-(1-7) may prevent cardiovascular damage in the setting of hypertension.(Giani *et al.*, 2011)

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