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Research Article

Potential Nephroprotective Effect of Valsartan in Renal Ischemia Reperfusion Injury Role of NF-KBP65 Pathway in Rat

BASIM I. MOHAMMAD AL-SHIBANI[1], MAYSAA ALI ABDUL KAHALEQ[2], MUNTHER ABOSAOODA[3], ALAA K. MOSA[4], MOHAMMED A. ABDULHUSSEIN[5], NAJAH R. HADI[6]
[1]Department of Pharmacology, Collage of Pharmacy, University of Al-Qadisiyah, Qadisiyah, Iraq
[2]Al Rasheed University College, Dept of Dentistry, Baghdad, Iraq
[3]College of Pharmacy, the Islamic University, Najaf
[4, 5, 6] Department of Pharmacology and Therapeutics, Collage of Medicine, Najaf, University of Kufa, Iraq
Corresponding Author: Maysaa Ali Abdul Khaleq
Email: maysaa_ali82a@yahoo.com
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ABSTRACT

Acute kidney injury (AKI) with all advances in nursing measures and therapeutic strategies, such as kidney transplantation and dialysis, mortality rate of patients with AKI is very high in past 30 years. Despite the pathophysiology of IRI is not totally understood, several critical mechanisms cause reversal of kidney damage have been demonstrated. In ischemic kidney and successive of generation of reactive oxygen species (ROS), re-oxygenation at reperfusion phase activate a cascade of destroying cellular responses causing to cell death, inflammation, and acute kidney failure. Valsartan is an Angotensin2 receptor blocker which is used as antihypertensive drug. It demonstrates organ protective effects in hypertension, attenuated renal injury possibly through its anti-inflammatory and antioxidant effects and it offer kidney protection as evidence by significant reduction in kidney injury score.

Objective : To study the potential Nephroprotective effect of Valsartan in RIRI

Materials & methods: After one week of acclimatization, the rat was randomly classified into four groups (6 rat in each) as follows:

1-IRI (control) group: rats subjected to the renal ischemia for 30 min by clamping renal artery and reperfusion for 2 hour.

2-sham group: rats underwent the same anesthetic and surgical procedure except clamping of bilateral renal artery.

3-control vehicle group: rats received 10 % dimethyl sulfoxide by I.P route and underwent renal ischemia for 30 min by clamping of renal artery and then 2hour reperfusion.

4-Valsartan treated group: rats pretreated with Valsartan 10 mg/kg I.P, 30 min before clamping of renal artery and then underwent renal ischemia for 30 min and then reperfusion for 2 hour. At the end of reperfusion time renal tissue and blood samples were collected. Blood samples used for measurement of IL1β, NF-KB p65, TLR4, NGAL and urea and creatinine for measurement of renal function. Renal tissue used for determination of histopathological changes.

Results: Renal IRI causes significant ($p \leq 0.05$) increase in tissue level of IL-1β, NGAL, TLR-4 and NF-KB p65 and serum urea, creatinine, pretreatment with Valsartan cause significant ($p \leq 0.05$) decrease in tissue level of IL-1β, NF-KB p65, TLR-4, NGAL and serum urea, creatinine, also cause significant reversal of tissue damage when compared with IRI group.

Conclusion: Pretreatment with Valsartan significantly decreases renal ischemia reperfusion injury in the rat via their pleiotropic effects as anti-oxidant, anti-inflammatory and anti- apoptotic activity.

Keywords: (RIRI), Valsartan

INTRODUCTION

Acute renal injury (AKI), also known as acute renal failure, is a global public health problem that affects millions of people, and it has become more prevalent in recent years. Several risk factors like age, race, genetic factor, hypertension, and diabetes are associated with AKI (1). Acute kidney injury has been largely studied in clinical and experimental animal model. The mechanisms of the pathogenesis and etiology of AKI are complicate and involve ROS, mitochondrial dysfunction, inflammation, Autophagy, apoptosis and necrosis. Inflammatory

responses are other critical part in the induction and exacerbation of the AKI. Despite inflammation is a crucial part of the body's immune system, extra stimulation of cytokine secretion and inflammatory cells cause serious injury to the renal parenchyma cells. Ischemia activates a great delivery of substance from the injured tissue (DAMPs), like heat shock proteins, hyaluronic acid, fibronectin and DNA that activate Toll-like receptors (TLR2, TLR4, and TLR5) the evolutionary maintain family of trans-membrane receptors that are a kind of protein recognition pattern (PRR) (2). Activation of (PRR) may activate production of pro-inflammatory cytokines and the death signaling pathway (3). When engaged TLRs activate the formation of a chemokine, such as TNF-α, IL-1β, IL-6 and chemokine formed by keratinocytes, further accompanied by macrophage and Neutrophils infiltration and pro-inflammatory cytokines (4). Transduction of the signals after TLR evocation related to many adaptive proteins, such as MyD88 which is the most critical one, leading in evocation of transcriptional factor NF-κB, that lead to subsequent formation of chemokine, pro-inflammatory cytokines (5)(4). Also TLR-2 and TLR-4 expressed by dendrite cells, macrophages and tubular epithelial cells. This expression is raised at the time of kidney IRI (6). TLR-4 lack on kidney parenchyma cells inhibited the raise of pro-inflammatory cytokines and chemokine formation and macrophages and Neutrophils aggregation in experimental RIRI models (4). Nuclear Factor Kappa B p65 data suggest it of the critical players in the pathogenesis of ischemia-reperfusion injury is the NF-κB pathway (7). Considerable evidence suggests NF-κB in formation of chemokine, cytokines, (ROS) also in regulation of anti and pro-apoptotic signaling, seemingly important in the pathogenesis of IRI (8-9). NF-κB act as transcription factor in inflammatory cells and tubular epithelial cell, associating the cell dying signaling pathway and coordinated inflammatory proposed in conception of necro-inflammation. It has been demonstrate NF-κB evocation in kidney tubular epithelial cell irritated tubular damage and aggravated inflammation in animal models of RIRI (10). Valsartan it is a strong, non- peptide tetrazole derivatives, with selectivity blocks Ang II receptor1 (11).It belongs to the family of Ang II type 1 receptor (AT) blocker and about 20000 fold higher affinity for it more than for Ang II type 2 receptor (AT1) (11). Selective AT1 receptor inhibitor exerts different pleiotropic effects, such as anti-apoptotic, anti-inflammatory and anti-oxidative effects (12). This action causes reduction in blood pressure; inhibits sympathetic outflow decreases vascular smooth contraction, improves renal function and also leads to the reduction in progression of atherosclerosis lesion (13). Valsartan blocked the TLR-4 and NF-κB expression. Valsartan plays a critical part in protecting against ischemic reperfusion injury. The possible defensive mechanism is due to anti-inflammation action through TLR-4 and NF-κB signaling pathways (14).

MATERIALS AND METHODS

Preparation of animals

24adult male of Swiss Albino rat (weighting 250-350 g, aged 10-12 weeks) were purchased from Animal Resource Center, College of Science University of Duhok. All experiments were approved by Animal Care and Research Committee of the University of Kufa. Animals were housed in animal house of University of Kufa, in a temperature controlled (24°C ± 2°C) room with alternative 12-hr light and 12-hr dark cycles and were allowed free access to water and diet until the start of experiment.

Ethical Statement

This study was accordance in exacting understanding with the suggestions in the Guide and Use of Laboratory animals association for Laboratory animal science. All animals' considerations and conventions were approved by animal care committee. All rats are sacrifice was performed under xylazine and ketamine mixture anesthesia.

Design of the study

The rats were randomized into 6 groups (6 rats each):
Rats were subjected to bilateral renal ischemia for 30 min by clamping and reperfusion for 2 hours (15-16) as following Valsartan administrated 10mg/kg dissolved in DMSO solution (17), administrated i.p 30 minute before RIRI. Selected biochemical and morphological parameters will be followed in the sham-operated animals and rat subjected to RIRI and pretreated with DMSO, Valsartan.
1-IRI (control) group (n=6), rats underwent 30 min bilateral renal ischemia and 2 hr reperfusion (15) (16).
2-Sham group (n=6), rats underwent same anesthetic and surgical procedures except for ischemia.
3-Vehicle group (n=6), rats pretreated with DMSO (18) by intraperitoneal injection 30 min before ischemia reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours.
-4Valsartan treated group (n=6), rats were pretreated with Valsartan 10 mg/kg (17) by intraperitoneal injection 30 min before ischemia

reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours.

Preparation of Valsartan

The product Valsartan is soluble in DMSO 20 mg/mL (clear solution) according to signaled rich package insert.

Experimental study model

Rats were weighed, anesthetized using an intra-peritoneal injection of ketamine in dose of 100 mg/kg and xylazine in dose of 10 mg/kg. Under sedation (5-10 min), rats were placed on its back, fixed their limbs and tail with stickers to ensure their stability during surgery. Hair in the chest area was shaved and the skin disinfected. The reflexes were checked through pinching the tail and hind feet to be sure that the rats were sufficiently anesthetized. By making midline laparotomy incision to expose the abdomen and to expose both renal pedicles, the intestine was retracted. Using the bilateral model of ischemia, then the renal pedicles was isolated where both renal artery and vein were clamped using a non-traumatic micro vascular clamps were positioned around the renal pedicles (19). Occlusion was confirmed by observing patched blanching of the entire kidney surface and changes the color of a kidney from red to dark purple after several minutes. The total time of clamp was 30 minute (20) and during this procedure, 1ml normal saline was administered into abdomen, then the abdomen was covered using warm and moist gauze to keep animals well hydrated. After 30 min, the clamps were removed from pedicles permitting renal blood flow restoration which represent the beginning of the reperfusion phase. The kidney was returned back to its position, then the abdominal cavity incision was sutured in two layers using 3interrupted sutures (21). Post-euthanized by deep anesthesia (22) and both blood and tissue samples were collected for analysis.

Collection and preparation of samples

Preparation of blood samples for measurement of renal function

At the end of the experiment, rats were anesthetized about (2.5-3ml) of blood was directly gathered from the heart. The sample of blood was placed in a plane tube at 37°C without anticoagulant, then it will centrifuged at 3000 rpm for 10 min, then serum obtained used for the determination of Urea and Creatinine (19).

Preparation of tissue for measurement of TLR4, NF-KBp65, IL1β, NGAL

Renal section taken and homogenized with a high intensity ultrasonic liquid processor in 1:10 (w/v) phosphate buffered saline that contained 1% Triton X-100 and a protease inhibitor cocktail .The homogenate was centrifuged at 3000 rpm for 20 min at 4°C (15) (23). The supernatant was collected for determination of TLR4, NF-KBp65, IL1β, and NGAL.

Tissue sampling for histopathology Analysis and damage scores

The kidney tissue sample was fixed in 10% formalin, dehydrated in alcohol series, cleared in xylene and embedded in paraffin block. The tissue slide sections were cut about 5- µm thick horizontal and stain with H and E then sent to histopathology's for histological examination. After fixation, an investigator who was blinded to the experimental treatment groups performed an evaluation of scores. Tissue sections were examined by light microscopy and graded for degeneration/necrosis (24) (25), using quantitative measurements for the assessing scoring system of tissue damage. The damage of tubule characterized as tubular epithelial swelling , loss of brush border, vacuolar degeneration , necrotic tubules, cast development; the degree of kidney injury was estimated at X40magnification, the score of histological changes in the kidney were evaluated as previously described by the following Criteria:

Score 0, represents normal
Score1, represent < 25% of damage tubules
Score2, represent 25%-50% of damage tubules
Score3, represent 50% -75% of damage tubules
Score4, represent >75% of damage tubules (26) (27)

Statistical analysis

Statistical analyses were performed using SPSS 24.0 for window. Inc. Data were expressed as mean ± SEM analysis of variance (ANOVA) Was used for the multiple comparisons among all groups followed by post –hoc tests using Bonferroni method, for the histopathological renal changes, the Mann-Whitney U was used to assess the statistical significance of difference between two groups, the Kruskal -Wallis test was used to assess the statistical significance of difference across multiple groups in total severity score in all tests $P \leq 0.05$ was considered statistically significant.

Effect of Valsartan on kidney markers following Renal IRI

Effect on IL1β

Renal IRI causes significant increase in mean of tissue level of IL1β (3890.69±72.24pg/ml, p value 0.001) when compared with sham group (198.18±8.49 pg/ml, p value 0.001).valsartan causes significant decrease in mean of tissue level

of IL1β (1138.66±15.20pg/ml, p value 0.001) when compared with IRI group.

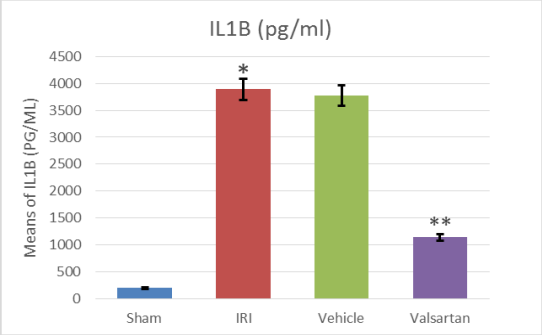

Fig. 1: Error bar chart show the effect of valsartan on tissue IL1β level following renal IRI , expressed as mean ± SEM pg/ml, n=6 in each group , *p value ≤ 0.05 when compared with the sham group , **p value ≤ 0.05 when compared with IRI group

Effect on NF-KBp65

Renal IRI causes significant increase in mean of tissue level of NF-KBp65 (4682.06 ± 114.04pg /ml, p value 0.001) when compared with sham group (120.45 ± 19.01pg/ml, p value 0.001). Valsartan cause significant decrease in mean of tissue level of NF-KBp65 (2200.21 ± 48.71pg/ml, p value 0.001) when compared with IRI group.

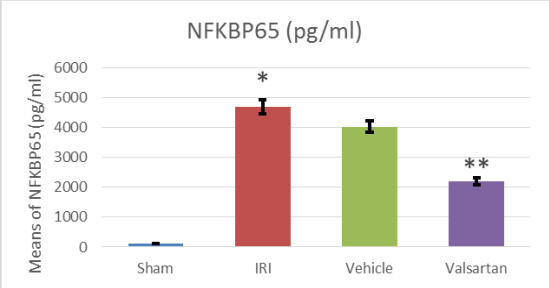

Fig. 2: Error bar chart show the effect of valsartan tissue NF-KBp65 level following renal IRI , expressed as mean ±SEM pg/ml, n=6 in each group , *p value ≤ 0.05 when compared with the sham group , **p value ≤ 0.05 when compared with IRI group

Effect on TLR4

Renal IRI causes significant increase in mean of tissue level of TLR4 (18.67 ± 0.40ng/ml, p value 0.001) when compared with sham group (1.06 ± 0.20ng/ml, p value 0.001). Valsartan causes significant decrease in mean of tissue level of TLR4 (14.61±0.40ng/ml, p value 0.001) when compared with IRI group.

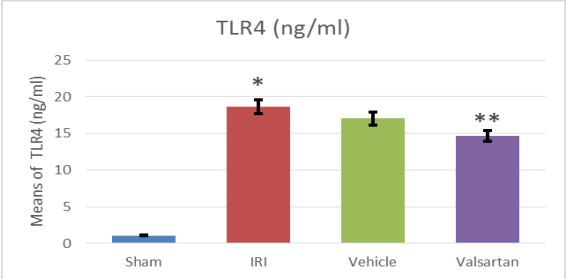

Fig. 3: Error bar chart show the effect of valsartan on tissue TLR4level following renal IRI, expressed as mean ± SEM ng/ml, n=6 in each group, *p value ≤ 0.05 when compared with the sham group, **p value ≤ 0.05 when compared with IRI group

Effect on NGAL

Renal IRI causes significant increase in mean of tissue level of NGAL (3459.39 ± 59.42pg/ml, p value 0.001) when compared with sham group (192.55 ± 16.15 pg/ml, p value 0.001), valsartan causes significant decrease in mean of tissue level of NGAL (2133.36 ± 6.23pg/ml, p value 0.001) when compared with IRI group.

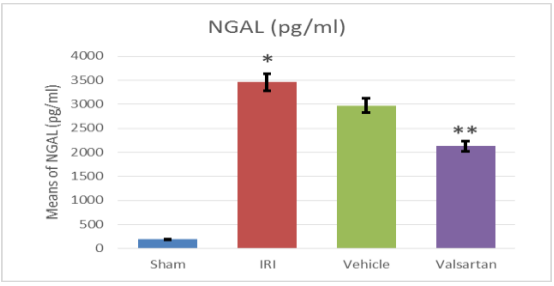

Fig. 4: Error bar chart show the effect of valsartan on tissue TLR4level following renal IRI, expressed as mean ±SEM pg/ml, n=6 in each group, *p value ≤ 0.05 when compared with the sham group, **p value ≤ 0.05 when compared with IRI group.

Effect of valsartan on blood urea and serum creatinine following renal IRI

Renal IRI causes significant increase in mean of both blood urea and serum creatinine (101.17 ± 2.29, 0.90 ± 0.02 mg/dl, p value 0.001, 0.001 respectively) when it compared with sham group (56.50 ± 0.56, 0.30 ± 0.03 mg/dl, p value 0.001, 0.001 respectively). Valsartan causes significant decrease in mean of both blood urea and serum creatinine (68.33 ± 2.11, 0.63 ± 0.04mg/dl, p value 0.001, 0.001 respectively) when it compared with IRI group.

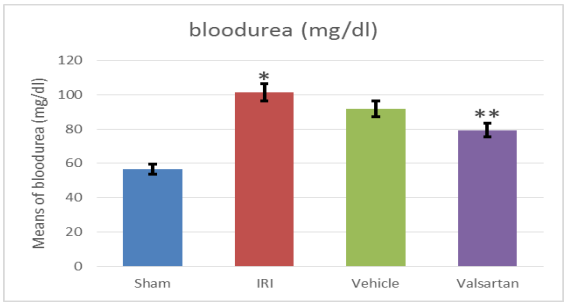

Fig. 5:Error bar chart show the effect of valsartan on blood urea following renal IRI, expressed as mean ± SEM mg/dl, n=6 in each group , *p value ≤ 0.05 when compared with the sham group , **p value ≤ 0.05 when compared with IRI group

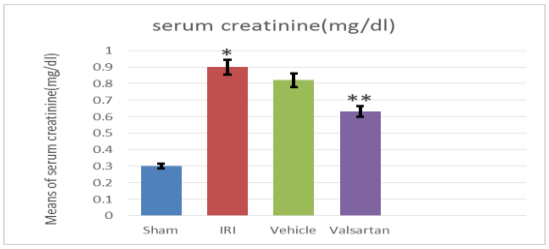

Fig. 6: bar chart show the effect of , valsartan on serum creatinine following renal IRI , expressed as mean ± SEM mg/dl, n=6 in each group , *p value ≤ 0.05 when compared with the sham group , **p value ≤ 0.05 when compared with IRI group

Histopathological findings

Figure (7) show the Histopathological score in the 4 experimental groups , IRI cause significant tissue damage (p value 0.001), pretreatment with valsartan cause reducing of tissue damage (p value 0.002,). Figure showed normal renal tubules without inducing IRI .figures showed significant tissue damage caused by IRI mainly apoptotic/necrotic renal tubules, figure showed how pretreatment with valsartan caused significant reduction in necrosis of renal tubules that caused by IRI.

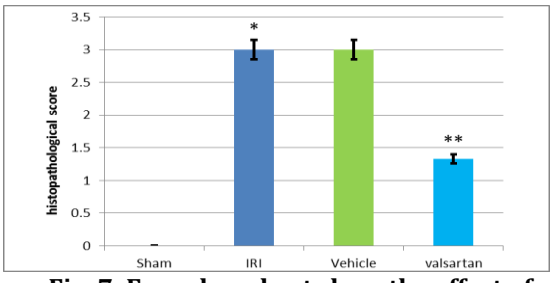

Fig. 7: Error bar chart show the effect of valsartan on Histopathological score of kidney injury following renal IRI , expressed as mean ±SEM; n=6 in each group, n=6 in each group , *p value ≤ 0.05 when compared with the sham group , **p value ≤ 0.05 when compared with IRI group

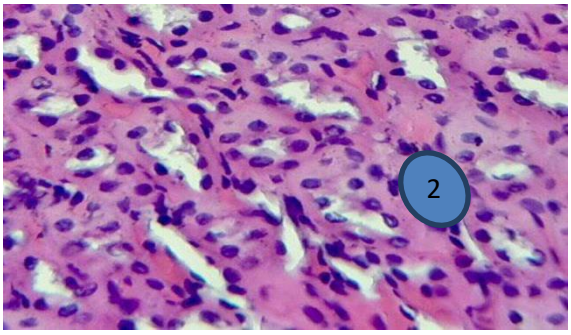

Fig. 8: Photomicrograph of adult male rat kidney with H and E staining at x40 magnification for the Sham-operated group (without induced IRI)

It does not show any morphological changes (illustrating severity score zero as it shows normal renal tubules) as marked (1), (2) normal renal tubules with intact glomeruli (red raw) and normal Baumann capsule (blue raw).

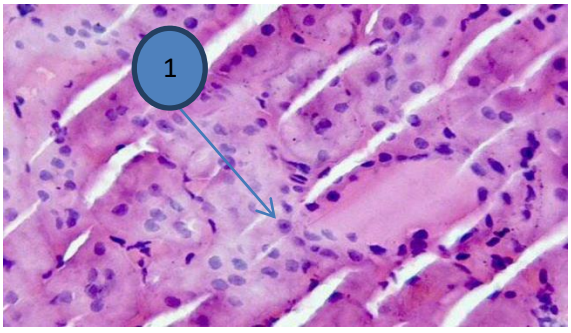

Fig. 9: Photomicrograph of not treated ischemic adult male rat kidney with H and E staining at x40 magnification for the control group (with induced IRI)

It shows morphological changes as cellular vacuolization, nuclear shading karryolysis and moderate to severe necrosis (illustrating severity score three as it shows tubular cellular swelling, loss of brush borders and eosinophilic cast) as marked (1) for eosinophilic cast.

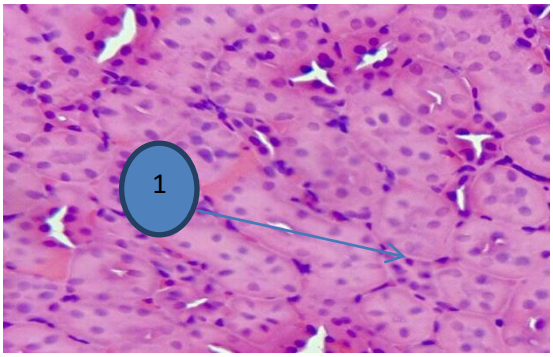

Fig. 10: Photomicrograph of adult male rat kidney with H and E staining at x40 magnification for the vehicle group (with induced IRI) that were pretreated with DMSO

It shows morphological changes as cellular vacuolization, nuclear degeneration and moderate to severe necrosis (illustrating severity score three as it shows tubular cellular swelling, loss of brush borders and eosinophilic cast formation) as marked (1) for tubular cellular swelling. The entire field shows mostly necrotic renal tubules.

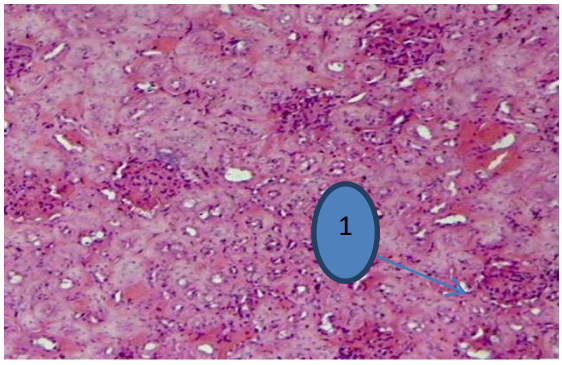

Fig. 11: Photomicrograph of adult male rat kidney with H and E staining at x40 magnification for the valsartan group (with induced IRI) and pretreated with Valsartan

In which morphological changes have not showed mostly as cellular vacuolization, nuclear degeneration, and moderate to severe necrosis (illustrating severity score one" ischemia cellular swelling in < 25% of tissue "as it shows normal renal tubules , less scattered apoptotic/necrotic cells with weak eosinophilic cytoplasm and without pyknotic nuclei)as marked (1) normal renal tubules. The entire field showed mild necrotic renal tubules.

DISCUSSION

Ischemia-reperfusion injury (IRI) is induced by a rapid temporary deterioration of the blood flow to the specific organ. IRI mostly is occurred with a powerful oxidative stress and inflammatory responses to the hypoxia and reperfusion that disrupts the organ function. RIR caused acute kidney injury (AKI) present with high morbidity and mortality rate in a broad range of injuries (28).

The effect of Renal I/R on renal function parameters (urea and creatinine)

In this study, I/R appears to have a higher level of urea and creatinine in control and control Vehicle group as compared with Sham group. These observations were in agreement with others studies (29) (30). Blood Urea and serum creatinine levels are mainly used as markers in evaluating renal functions. Significantly increase in urea and creatinine levels are indicators of AKI (31).This result also agrees with those reported by (32) which revealed that I/R caused a critical rise in serum levels of creatinine and alanine aminotransferase after 30 min ischemia and 4 hr reperfusion.Data indicates that the untreated IRI shows a higher level of urea and creatinine in control group as compared with the sham group (33). Also studies indicate that the ischemia reperfusion group shows significantly higher concentration of creatinine and urea after 45 min ischemia, 24 hr reperfusion in a rat model (34).

The effect of Valsartan on renal function parameters (urea and creatinine)

In this study, Valsartan significantly lowers the levels of urea and creatinine as compared with the control group, demonstrating the preservation of renal function. This results is consistence with (35)(36) as their results indicate a significant diminish in the levels of urea and creatinine in valsartan treatment group.

Effect of Renal IR on Inflammatory Mediators (IL-1β and NF-κB p65)

This research proved that there is a significant increase in inflammatory mediators (IL-1β and NF-κB p65) were found in the control and vehicle group as compare with the sham group, additionally the present study mounted that the Inflammatory Mediators had been proven to response key roles within the pathophysiology of ischemia/reperfusion injury(37). IL-1β is considered as chemo-attractant that recruits leukocytes to the area of the renal inflammation which causes eventually to the kidney damage. During the early phase of IRI, Large amounts of reactive oxygen species and nitrogen intermediates and pro-inflammatory cytokine such as (IL-1β and TNFα) are produced by M1machrophage and drive a polarized Th1 immune response which contributes to injury (38). NF-KB plays a critical role in the pathogenesis of RIRI (39). It is a transcriptional factor that is activated by cytokines and chemokine after AKI (40). Data showed that levels of (NF-κB p65) were increased in I/R group when the rats subjected to 30 min ischemia, 2 hours reperfusion (15).

The effect of Valsartan on Inflammatory mediators (IL-1β and NF-κB p65)

The present study reveals that there is a significant decrease in renal level of (IL-1β and NF-κB p65) for Valsartan pretreated group as compared to control group. These findings suggest that Valsartan performs a fundamental role within the protective effects on renal I/R damage. This protection mechanism is likely because of its anti-inflammation function through TLR-4 along with NF-κB signaling pathway (14) (41).

The effect on Renal TLR-4 level

In the present study, a significant increase in tissue TLR-4 level has been found in the control group as compared with the Sham group. Moreover, the present study establishes that the TLR-4 signaling pathway plays an important role in the renal IRI-associated inflammation and apoptosis. This result is in agreement with those reported by (42), their results suggest that the activation of TLR-4 signaling contributes to the pathogenesis of renal I/R injury. Data showed that the TLR-4 is markedly up-regulated in both experimental and human acute renal allograft rejection (43).

The effect of Valsartan on TLR level

The present study find out that valsartan pretreated causes a significant lower level of TLR-4 in Valsartan treated group as compared with the control vehicle group. These findings suggest that Valsartan plays a fundamental role in the possible due to its anti-inflammation function via NF-κB signaling pathway. Valsartan administration in AKI rats largely enhances catalase, glutathione peroxidase superoxide dismutase, and glutathione amounts. but decreases the amounts of lipid peroxidation. Valsartan significantly decreases the severity of the renal lesions, necrosis and renal tubular injury. It reduces NF - κB and TLR4 mRNA formation by >50% and their protein amounts by>40%. So, Valsartan administration prevents glycerol-induced functional and pathological injury to the kidney in a concentration-dependable manner. Data suggest that Valsartan protects rat kidney tissue by down regulating the TLR-4 and NF- κB expression (44). Valsartan has the possible protection mechanism due to its anti-inflammation function via TLR-4/NF-κB signaling pathway and a critical role in the defensive action (14).

The effect of Renal IR on Renal Injury marker NGAL

It has been observed, that there is considerable increase in tissue level of NGAL found in the induced non treated group as compared with Sham group. This result is consistent with other studies that predicated NGAL as a biomarker of AKI (45). After acute kidney injury NGAL is early identified as one of the earliest markers of renal damage after ischemic injury in animal models, therefore, renal function can be monitored by using NGAL biomarker (46) (47). These effects recommend that the level of NGAL is sensitive and regarded as particular markers of acute kidney injury in animals (48).

The effect of Valsartan on Renal Injury marker NGAL

Our study shows that there is a significant lower in the renal level of (NGAL) in Valsartan pre-treated group as compared with control vehicle group.Best of our knowledge, there is no data available about the effect of Valsartan on renal injury marker NGAL in renal ischemia reperfusion injury. The results of our study show that effect of Valsartan on renal injury marker NGAL is due to the anti-oxidant effect.

The effect of renal IR injury on renal parenchyma

IRI histological examination shows more tissue injuries, including dilatation of the Bowman's capsule lack of brush borders, necrotic areas, vacuolization dilatation of renal tubule and glomerular modifications. These changes are in agreement with some other studies such as (15) who showed that there was significant swelling vacuolar degeneration in mitochondria, shrinkage of microvilli, disappearance of brush borders, segmental foot method fusion, and glomerular basement membrane thickening in kidneys.

The effect of Valsartan on Renal Parenchyma

Treatment of rat with Valsartan lowers renal injury drastically as compared with the control group. The total severity score mean of this group confirms a moderate renal injury. The study verifies that Valsartan, which administered before the renal I/R damage, prevents renal injury through histopathological parameters. The results of the present study agrees with other studies indicated that the Valsartan treatment group had statistically significant histopathological tubular vacuolization, lack of brush border and tubular dilatation (49-50).

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