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Evaluation the Nephroprotective Effect of Rosuvastatin through Activation of NrF2\HO1 Pathway in Renal Ischemia Reperfusion Injury in Rats

Esraa A. Alyasiry[1], Ahmed M. Abdul Hameed[2], Huda J. Merza[3]

[1]Department of Pharmacology and Therapeutics, College of Medicine, Jabir Ibn Hayyan Medical University, Najaf, Iraq \ ORCID: 0009-0003-4352-8745 \ Email: esraa.ahmed@jmu.edu.iq
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Jabir Ibn Hayyan Medical University, Al Najaf Al-Ashraf, Iraq \ ORCID ID: 0000-0002-8745-4616 \ Email: ahmed.m.abdulhamid@jmu.edu.iq
[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Jabir Ibn Hayyan Medical University, Al Najaf Al-Ashraf, Iraq Email: huda.j.merza @jmu.edu.iq
*Corresponding Author:
Ahmed M. Abdul Hameed
Department of Pharmacology and Toxicology, Faculty of Pharmacy, Jabir Ibn Hayyan Medical University, Al Najaf Al-Ashraf, Iraq \ ORCID ID: 0000-0002-8745-4616 \ Email: ahmed.m.abdulhamid@jmu.edu.iq.

ABSTRACT

Background: When ischemic tissues are rapidly reperfused by blood, the endothelial layer of capillaries creates reactive oxygen species (ROS), which worsens the NFκB pathway and inflammatory process. Ischemia also causes structural and functional alterations in the microvascular system. Numerous components of inflammation are produced, including NFκB, IL-1β, and KIM1. The main cause of morbidity and mortality in some disorders, such as sepsis, MI, and AKI, seems to be ischemia reperfusion damage.

Objective: This study is occurred to check the effectiveness of Rosuvastatin in attenuating renal injury during IR, possibly through activation of Nrf2\HO1 signaling pathway.

Method: Twenty Wister Albino rats were divided into four equal groups at random, (N=5): Sham: Rat laparotomies were performed without ischemia. Control: Rats undergone laparotomy with bilateral RIRI for 30-minute following two hours of reperfusion. Vehicle: Rats given an intraperitoneal injection of DMSO three days before induction of RIRI. Rosuvastatin: Rats received an intraperitoneal injection of Rosuvastatin three days prior to RIRI.

Results: NFκB, IL-1β, and KIM-1 tissue levels were significantly lower in the sham than in the vehicle and control; the results also revealed that Rosuvastatin had significantly lower levels of KIM-1, NFκB, IL-1β than in the vehicle and control; and the histopathology demonstrated that Rosuvastatin could significantly reduce kidney damage compared to the vehicle and control. On the other hand, tissue content of Nrf2\HO1 was elevated in Rosuvastatin group compared to I\R group.

Conclusion: This study concluded that Rosuvastatin significantly reduced RIRI damage in rats through activation of Nrf2\HO1 antioxidant signaling pathway.

KEYWORDS: Rosuvastatin, RIRI, Nrf2, HO1, NFκB..

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INTRODUCTION

Abbreviations

AR (Rosuvastatin), I/R (ischemia reperfusion), Nrf2 (Nuclear factor erythroid 2–related factor 2), HO-1 (Heme oxygenase-1), KIM-1 (Kidney Injury Molecule-1), IL-1β (Interleukin-1 beta), DMSO (dimethylsulfoxide), RIRI (Renal Ischemia Reperfusion Injury).

Introduction

When an organ experiences a temporary reduction or suspension of blood flow, followed by a restoration of perfusion, the net effect of an inflammatory process is ischemia reperfusion injury (IRI) **(Jallawee and Janabi, 2024).** Numerous clinical scenarios, involving sepsis, drug-induced ischemia, shock, heart and vascular surgery, and organ transplantation., can result in IRI **(Kanagasundaram, 2015).**

The pathophysiology of renal IRI is significantly influenced by inflammation and the immune system. Initial kidney injury and long-term structural changes, such as interstitial fibrosis or healing, are believed to be caused by immune system engagement **(Jallawee and Janabi, 2024).** By recruiting leukocytes, up-regulating adhesion molecules, and producing mediators such as cytokines, chemokines, ROS, and eicosanoid, inflammatory cells can exacerbate kidney damage **(Yahiya *et al.*, 2023).**

IL1β is one significant cytokine examples. One of the principal pro-inflammatory mediators or cytokines, **(Olszewski *et al.*, 2007).**

Nuclear factor kappa B (NF-kB) regulates the processes of apoptosis, immunology, inflammation, and survival **(Girard *et al.*, 2009)**. A number of important inflammatory cytokines, such as TNF-α and IL1β, cause NF-kB to become active and take part in the effector phase of inflammation **(Tak and Firestein, 2001)**. In a way that is dependent on NF-kB, ischemia/reperfusion triggers the synthesis of TNF-α. TNF-α then attaches to its receptor to trigger NF-kB activation, resulting in a positive feedback process of NF-kB regulation. This signaling loop is essential to RIRI development **(Zhang and Sun, 2015)**.

To assess renal function and ascertain whether GFR is normal, several functional markers, or clinical laboratory tests, are available. Among these specific functional assays is Kidney Injury Molecule-1 (KIM1). Kidney Injury Molecule-1, a transmembrane glycoprotein, is now a crucial biomarker for identifying kidney damage, particularly acute kidney injury (AKI). KIM1 expression is low in healthy kidneys but increases considerably in proximal tubular epithelial cells following renal injury **(Brilland *et al.*, 2023).** This increase makes KIM1 a valuable marker for the early diagnosis and prognosis of AKI, according to Sabbisetti et al. 2014 **(Sabbisetti *et al.*, 2014).** The significance of the biomarker is highlighted by KIM1's ability to predict AKI before more well-known indicators, such as serum creatinine, show discernible changes. For this reason, KIM1 is useful in clinical settings for kidney damage management and timely intervention **(Han *et al.*, 2002)**.

By increasing the formation of reactive oxygen species (ROS), oxidative stress (OS) is a significant route that contributes to the pathophysiology of IRI **(Jiang *et al.*, 2015).** ROS are small, extremely reactive chemicals that could be dangerous. They cause lipid peroxidation, enzyme inactivation, glutathione oxidation, the production of organic radicals, and cell death by reacting with biological components including DNA, thiols, carbohydrates, and the lipids and proteins that make up the cell membrane. Nonetheless, ROS, particularly H2O2, can benefit tissues mostly through their typical role in cell signaling. As a result, a cell's ROS levels need to be strictly controlled **(Korkmaz and Kolankaya, 2010).**

A master regulator of xenobiotic metabolism and the antioxidant response by controlling a large number of genes related to Phase II detoxification and antioxidants. **(McMahon *et al.*, 2001)**. Nrf2 shields cells against external pollutants, radiation, endogenous chemicals, reactive oxygen species (ROSs), and xenobiotics from diet or the environment. As a result, Nrf2 pathway activation may be a viable chemoprevention technique. **(Huang *et al.*, 2015)**. The 605 amino acid string that makes up Nrf2, a transcription factor belonging to the Cap'n'collar (CNC) family, is separated into seven highly conserved functional domains called Neh1–Neh7. The cytoplasmic location of Nrf2 is controlled by the Neh5 domain, whereas the N-terminal domain affects its stability and ubiquitination by its negative regulator Keap1. **(Krajka-Kuźniak, Paluszczak and Baer-Dubowska, 2017)**

Heme oxygenase-1 (HO-1, HMOX1, EC 1.14.99.3), is a 32 kDa protein that can be induced and catalyzes the rate-limiting process of oxidative heme breakdown. Three bioactive products are produced from heme during this process: free iron, carbon monoxide (CO), and biliverdin, which is quickly transformed into bilirubin and is essential for oxidative stress, inflammation, and apoptosis. **(Wunder and Potter, 2003)**. Like other antioxidant proteins, Nrf2 directly regulates the expression of the HO-1 enzyme-coding HMOX1 gene. According to a number of in vitro and in vivo studies, Nrf2-mediated HO-1 expression is essential for the anti-inflammatory action. **(Saha *et al.*, 2020).**

Rosuvastatin is a common cholesterol-lowering drug because of its strong inhibitor of HMG-CoA reductase. Inhibiting HMG-CoA reductase, an enzyme required for cholesterol production, is its main pharmacological activity. Rosuvastatin effectively lowers the liver's production of cholesterol by blocking the enzyme that changes HMG-CoA into mevalonate, a precursor of cholesterol **(Karr, 2017)**. Through the upregulation of low-density lipoprotein (LDL) receptors on hepatocyte surfaces, this inhibition enhances the removal of LDL cholesterol from the bloodstream **(Sri Chandini, 2016)** . In addition to reducing low-density lipoprotein (LDL), Rosuvastatin is advantageous for a number of lipid indicators. It contributes to a better overall lipid profile by raising HDL cholesterol and lowering triglycerides **(Martin *et al.*, 2003)**. On its own, Rosuvastatin has also been demonstrated to lower inflammatory markers, including high-sensitivity C-reactive protein (hs-CRP), which is a risk factor for cardiovascular events **(Ridker *et al.*, 2008)**.

MATERIAL AND METHOD

Research Site and Ethical Considerations

The research was conducted at the Toxicology and Pharmacology Department\ Faculty of Pharmacy \ University of Kufa and in Middle Euphrates Unit for Cancer Researches \ Faculty of Medicine \ University of Kufa. The study was accepted by Committee center of Bioethics in the University of Kufa and its representative in Faculty of Pharmacy. Whole procedures were done according to the recommendations of the Committee.

Study Protocol

20 mature Wister Albino rats weighing between 220 and 350 grams and 20 to 25 weeks of age were used in this investigation. They were obtained from the Ministry of Health's Centre of Control and Pharmaceutical Research. Before the operations began, the animals were kept in the Faculty of Science/University of Kufa's animal house for 14 days at a temperature of 20–25 degrees Celsius, 60–65% humidity, and a 12-hour light/dark cycle. The rats also had unrestricted access to food and water. Rats were randomly assigned to four equal groups for this investigation, with seven rats in each group. The groups were as follows:

1. **Sham group:** For the same amount of time, all seven rats received the same anesthetic and procedures to treat ischemia and reperfusion in the absence of ischemia potentiation of reperfusion. Blood samples and renal tissues were gathered.
2. **Control group:** following a 30-minute bilateral renal ischemia and a median laparotomy performed under anesthesia on all seven rats, renal tissues and blood samples were taken two hours following reperfusion **(Yahiya *et al.*, 2023).**
3. **Vehicle group:** Three days prior to the induction of RIRI **(Chen *et al.*, 2018),** all seven-albino rats received an intraperitoneal injection of DMSO. They then experienced bilateral renal ischemia for 30 minutes and reperfusion for two hours **(Alaasam *et al.*, 2024; Hameed *et al.*, 2021).** At last, both kidneys were removed.
4. **Rosuvastatin group:** Three days before to the induction of RIRI **(Chen *et al.*, 2018)**, all seven-albino rats received an intraperitoneal injection of Rosuvastatin 10 mg/kg **(Chiorescu *et al.*, 2018)** . They then experienced bilateral renal ischemia for 30 minutes and reperfusion for two hours **(Alaasam *et al.*, 2024;Hameed *et al.*, 2021).** At last, both kidneys were removed.

Renal ischemia Reperfusion Injury Rat Model

Using intraperitoneal injections of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride, all of the rats were put to sleep. A heat plate was used to maintain the rats' body temperature at roughly 37 ^{0}C. After shaving and cleaning the abdomen region with an antiseptic to prevent infection, the midline incision was made, exposing the renal pedicles by first slicing the abdominal skin and then the abdominal muscle. Clamping of left and right renal pedicles for half hour with no-traumatic vascular clamps. One milliliter of warm sterile saline was infused into the peritoneal cavity to preserve good hydration. After ischemic time ending, the clamps were removed for reperfusion, suture, and cover the wound by sterile gauze damping with normal saline to avoid dehydration.

The suture was opened after the two-hour reperfusion period, and approximately three milliliters of blood were extracted from the heart. This was followed by a bilateral nephrectomy, during which the kidney was cleaned of blood using precooled phosphate buffer saline (PBS). ended up sacrificing the rat by puncturing its heart. **(Tweij et al., 2022).** The left kidney was divided in half sagittaly. For biomolecular analysis, the first half was stored in a deep freezer. For histological and immunohistochemical evaluation, the second half was placed in 10% formalin and subsequently embedded in paraffin..

Preparation of the Drug

The drug was prepared immediately before using by dissolved in DMSO **(Solubility: In DMSO: 20 mg/ml)** as descripted by manufacturer (Medchemexpress).

Assessment of Tissue, NFκB, IL-1β, HO1, and KIM1

Following the division of the frozen kidney part into small pieces and a cold PBS wash, the tissue was weighed and first homogenized using a mortar and pestle with 1:10 (W/V) 0.1 M of precooled PBS (PH 7.4), have one percent Triton 100X and one percent of the protease inhibitor mixture. **(Jaya Dwivedi *et al.*, 2017; Stokman *et al.*, 2010).** For optimal homogenization, the homogenate was sent through a high-intensity ultrasonic liquid processor to further break down the cell membranes. Finally, the homogenate was centrifuged for ten minutes at 4 oC and 10,000 rpm. NFκB, IL-1β, HO1, and KIM1 levels were measured using the supernatant and an ELISA Sunlong kit.

Histopathological Analysis

The left kidney was drained, cleaned, and then fixed in paraffin before being sliced into 5-micrometer-thick pieces using a rotary microtome. The tissue section was then fixed on slides, stained with hematoxylin and eosin dye, and covered to get ready for microscopic inspection. Two skilled pathologists assessed renal tissue damage blindly while taking into account six randomly chosen fields.A scale design was used to classify the sections in order to evaluate the extent of renal injury, including vascular and tubular necrosis degeneration, eosinophilic cast formation, loss of brush boundary, swelling of renal epithelial cells, and desquamation of epithelial cells into the lumen. Five scores were utilized in the scoring system: 0 for normal kidney tissue, 1 for less than 25% renal damage, 2 for 25%–50% kidney damage, 3 for 50%–75% kidney damage, and 4 for more than 75% kidney damage. **(Shi *et al.*, 2019).**

Immunohistochemistry assessment

The measurement of Nrf2 in kidney tissue was done using immunohistochemistry. The immunostaining technique was used to stain 5μm paraffin embedded sections. In short, sections were deparaffinized, rehydrated, subjected to retrieval buffer to restore the antigen, and 3% H2O2 was added to block endogenous peroxidase activity. The sections spent the night at 4 oC treated with Nrf2 monoclonal antibody (1:200, bioassay). Following washing, the slices were incubated with a conjugated secondary antibody for one hour, then cleaned and exposed to horseradish peroxidase for thirty minutes. Following that, the slices were incubated for eight minutes with fresh 3, 3′-diaminobenzidine. Lastly, the counterstain was hematoxylin stain. Next, use a microscope to view the staining. By multiplying the intensity and percentage of the stained area, the H-score method (which ranges from 0 to 300) was used to determine the protein expression of Nrf2. A score of 0–3 was assigned to the stain intensity: 0 denoted no staining, 1 weak staining, 2 moderate staining, and 3 severe staining. The percentage of stained cells was ranked between 0% and 100%.**(Rajarajan *et al.*, 2020).**

Statistical Analysis

Microsoft Windows Inc.'s GraphPad Prism version 8.0.2 was used for statistical analysis. The mean±SD was used to display the data. One-way analysis of variance, or one-way ANOVA, and post hoc. Tucky test were utilized to perform multiple comparisons

across all groups. All comparisons and tests were considered statistically significant if $P < 0.05$.

RESULTS

Rosuvastatin Improve Renal Function Parameter

Rats in the vehicle and control groups showed a markedly higher tissue level of KIM1 than the sham group. Comparing the rosuvastatin pretreatment group to the control and vehicle groups, the kidney tissues content of KIM1 was considerably lower (Figure 1).

Rosuvastatin Decreased the Inflammatory Marker in Renal Tissue (IL1β)

The kidney homogenate of control and vehicle rats showed considerably higher levels of protein expression of the inflammatory mediator IL1β than sham rats. Rats given 10 mg/kg of AR intraperitoneally for three days in a row showed a substantial decrease in IL1β expression when compared to control and vehicle rats (Figure 2).

Rosuvastatin Decreased the (NFκB) in Renal Tissue

The kidney homogenate of control and vehicle rats showed considerably higher levels of protein expression of the inflammatory mediators (NFκB) than sham rats. Rats given 10 mg/kg of AR intraperitoneally for three days in a row showed a significant decrease in NFκB expression when compared to control and vehicle rats (Figure 3).

Rosuvastatin Attenuated Oxidative Stress through Increasing HO1 Level in Renal Tissue

In our experimental study, we demonstrated that the sham group's renal tissue level of HO1 was considerably ($p < 0.001$) greater than the levels in the vehicle and control groups. Compared to the control and vehicle groups, the AR treatment group's renal tissue level of HO1 was considerably ($p < 0.001$) greater (Figure 4).

Rosuvastatin Upregulated Nrf2 Expression

In this study, we showed that the sham group's renal tissue Nrf2 expression was considerably ($p < 0.001$) higher than that of the control and vehicle groups. The renal tissue Nrf2 level in the AR pretreatment group was considerably ($p < 0.001$) higher than that of the control and vehicle groups (Figure 5 and 6).

Rosuvastatin Minimized Kidney Injury

Histopathological analysis revealed that the sham group had no kidney damage. Compared to the sham group, there were more damaged tubules and cell dilatation in the control and vehicle groups ($P < 0.001$). Compared to the control and vehicle groups, the AR-pretreated group displayed minimal histological alteration ($P < 0.001$) (Figure 7 and 8).

DISCUSSION

Effect of rosuvastatin on Kidney Injury Molecule-1 (KIM1)

According to this experimental work, Rosuvastatin pretreatment before ischemia stimulation significantly ($P < 0.001$) lowers the amount of KIM1 in renal tissues when compared to the vehicle and control groups. According to this study, after renal IRI development, rosuvastatin preserves renal tissues and function parameters in a rat model. This outcome is consistent with previous studies. Rosuvastatin treatment shielded the kidney from oxidized LDL damage in chronic kidney disease (CKD) and was shown to reduce the level of KIM1 in a recent experimental research on CKD rats **(Sung *et al.*, 2022)**.

Rosuvastatin's Impact on the Kidney Parenchyma

This study shown that, in comparison to the vehicle and control groups, the degree of kidney injury is significantly ($P < 0.001$) reduced when Rosuvastatin, an HMG-CoA reductase inhibitor, is administered prior to ischemia induction. The vehicle and control groups' mean score intensity indicated severe kidney damage, while the Rosuvastatin-pretreated group's mean score intensity indicated mild to moderate impairment. Our findings are in line with those of other studies.

According to recent research by Shafik et al. (2023), when Rosuvastatin is administered concurrently to a group of rats that are taking Colistin for six days in a row, it can cause renal injury, prevent the severity of Colostin-induced nephrotoxicity, preserve the renal parenchyma, and lessen the severity of tubular injury, necrosis, cast formation, and tubular dilatation compared to the untreated control group, which exhibits all these histological changes in a high degree **(Shafik *et al.*, 2023)**.

Effect of Rosuvastatin on the Inflammatory Mediator (NF-κB and IL-1β)

This animal study found that premanagement with Rosuvastatin can significantly ($P < 0.001$) lower the concentration of inflammatory molecules (NF-κB and IL-1β) in ischemic renal tissues, in contrast to the levels of cytokines linked to inflammation in the vehicle and control groups. So Rosuvastatin can reduce inflammation in renal tissues that have undergone ischemia and reperfusion.

These results are consistent with other studies. Rosuvastatin can lower IL-1β, and NF-κB levels in rats given Cisplatin to cause nephrotoxicity, according to an experimental study by Saad et al. (2024) **(Saad *et al.*, 2024)**. An additional experimental study demonstrated that administering Rosuvastatin at two different doses to a group of rats receiving three intraperitoneal injections of thioacetamide per week for six weeks to induce liver fibrosis can lower the levels of IL-1β, and NF-κB in the liver tissues compared to the group receiving thioacetamide alone **(Ghaith *et al.*, 2023)**.

Effect of Rosuvastatin on the Nrf2\HO1 Expression

In this experimental investigation, we showed that both the control and vehicle groups had considerably ($p < 0.001$) lower levels of renal tissue Nrf2 expression than the AR group. This finding implies that rosuvastatin has an anti-oxidative impact on renal tissues that have experienced reperfusion and ischemia. These findings are in line with previous research; rosuvastatin corrected oxidative biomarker alterations brought on by a high-salt and cholesterol diet (HSCD) and demonstrated an excellent theoretical affinity for Nrf2. An increase in Nrf2 nuclear translocation was detected by western blot and immunohistochemistry analysis. The Nrf2 pathway may be protective against the degenerative processes caused by HSCD, according to these findings. **(Husain *et al.*, 2018)**. According to our findings, Rosuvastatin's protective effect is mediated through the activation of Nrf2, which leads in the induction of HO1 and the reduction of oxidative damage. Similar protective effects have been shown in diabetic nephropathy and splenic damage models **(Fahim *et al.*, 2023; Heeba, Ali and El-Sheikh, 2022)**

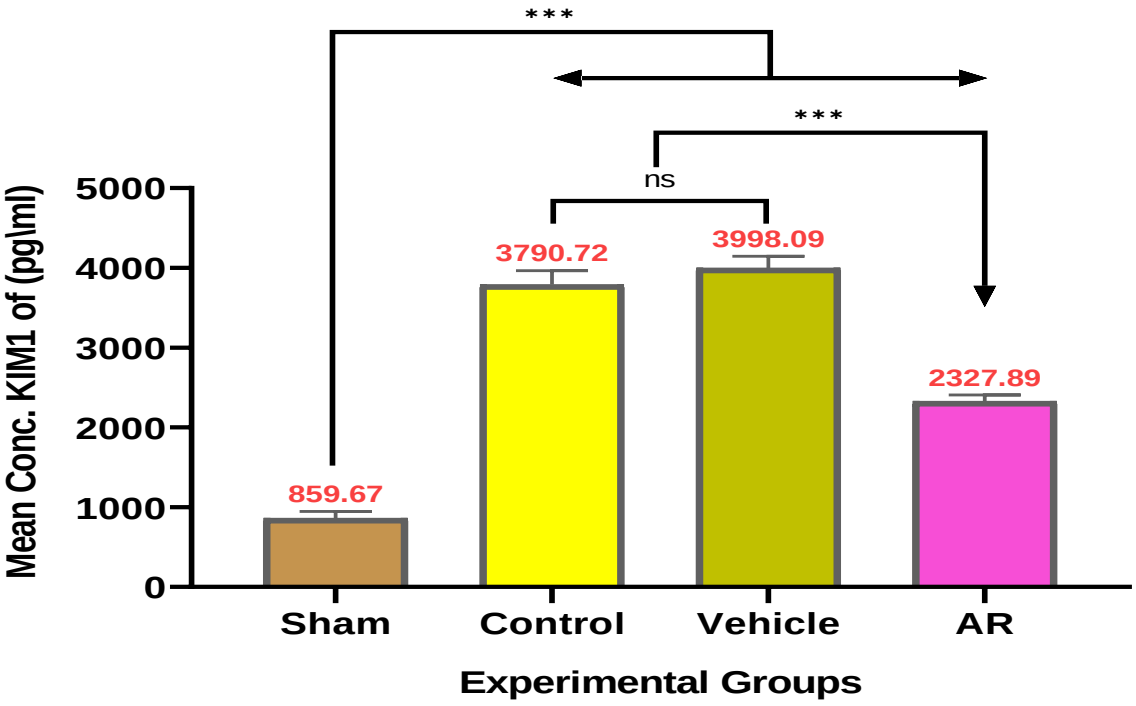

Figure (1): Represents the statistical analysis of KIM1 concentrations mean (pg/ml) in renal tissues in the four experimental study groups (No of rats = 5 in each study group).

Sham group vs. vehicle & control groups, ***P.value < 0.001AR vs. vehicle & control groups, ***P.value < 0.001

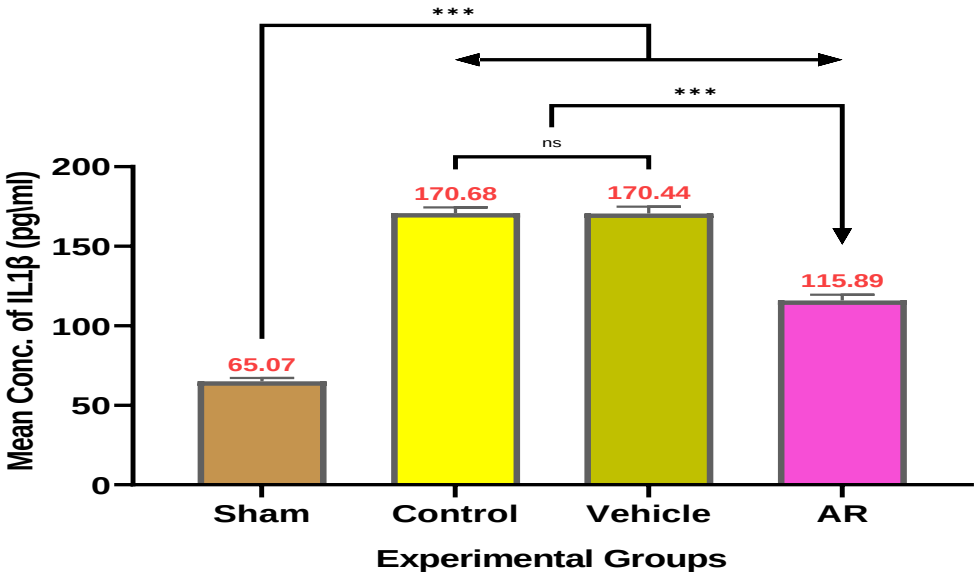

Figure (2): Represents the statistical analysis of IL-1β concentrations mean (pg/ml) in renal tissues in the four animal study groups (No of rats = 5 in each study group).

Sham group vs. vehicle & control groups, ***P.value < 0.001
AR vs. vehicle & control groups, ***P.value < 0.001

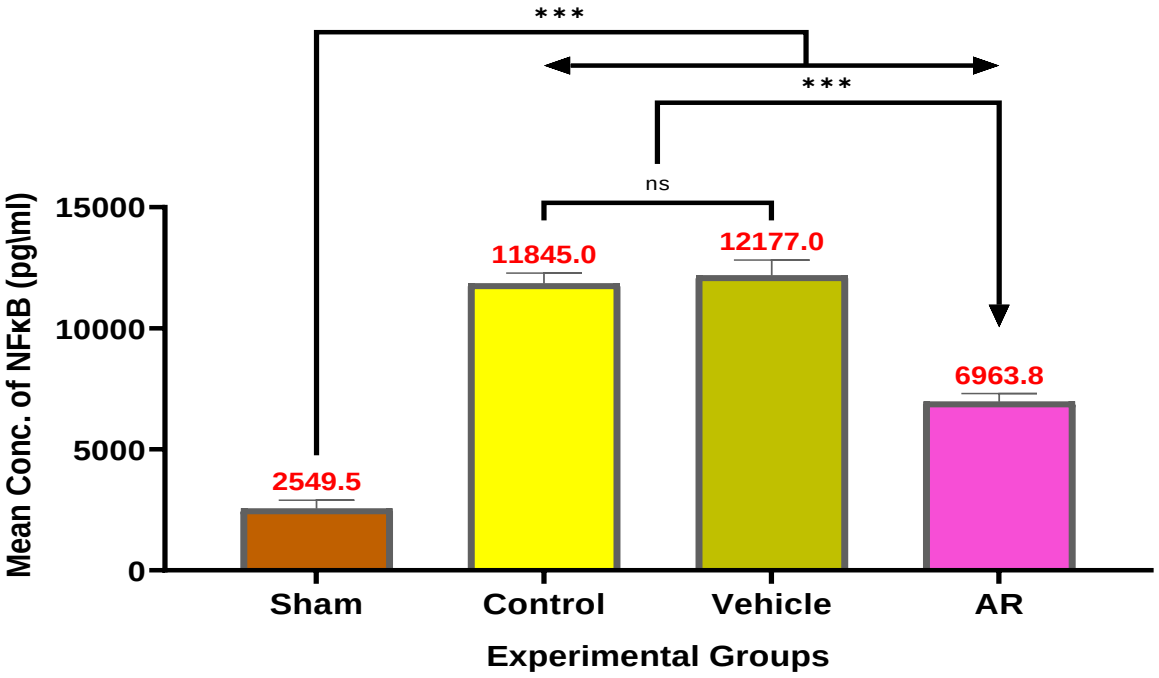

Figure (3): Represents the statistical analysis of NFkB concentrations mean (pg/ml) in renal tissues in the four animal study groups (No of rats = 5 in each study group).

Sham group vs. vehicle & control groups, ***P.value < 0.001
AR vs. vehicle & control groups, ***P.value < 0.001

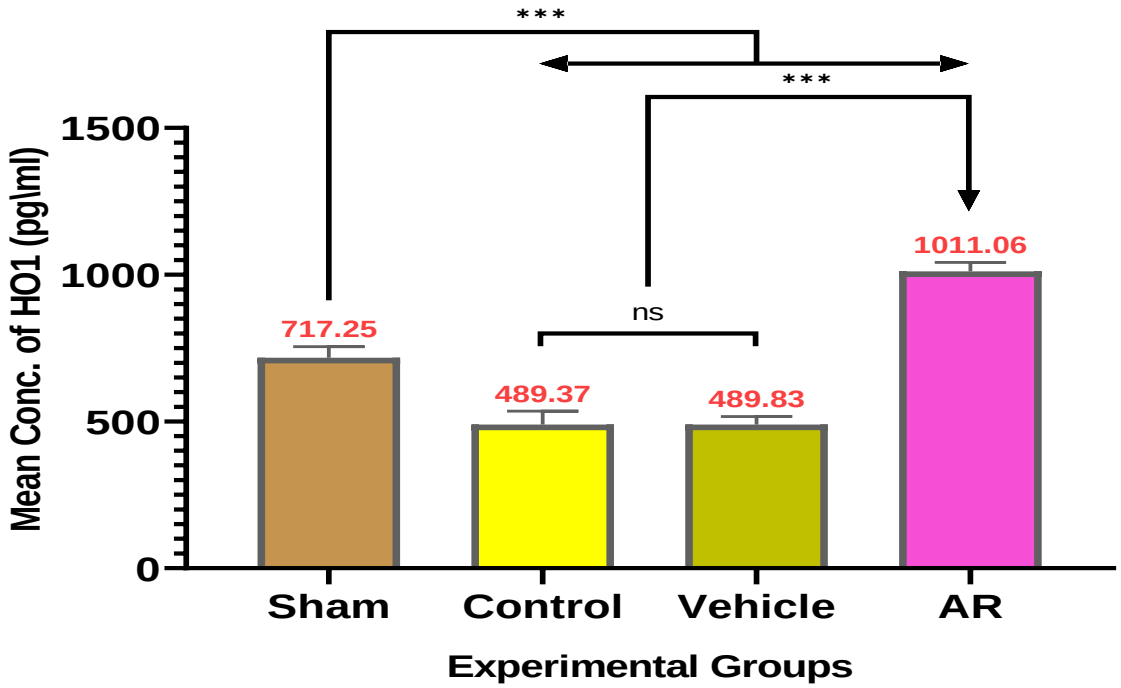

Figure (4): Represents the statistical analysis of HO1 concentrations mean (pg/ml) in renal tissues in the four animal study groups (No of rats = 5 in each study group).

Sham group vs. vehicle & control groups, ***P.value < 0.001
AR vs. vehicle & control groups, ***P.value < 0.001

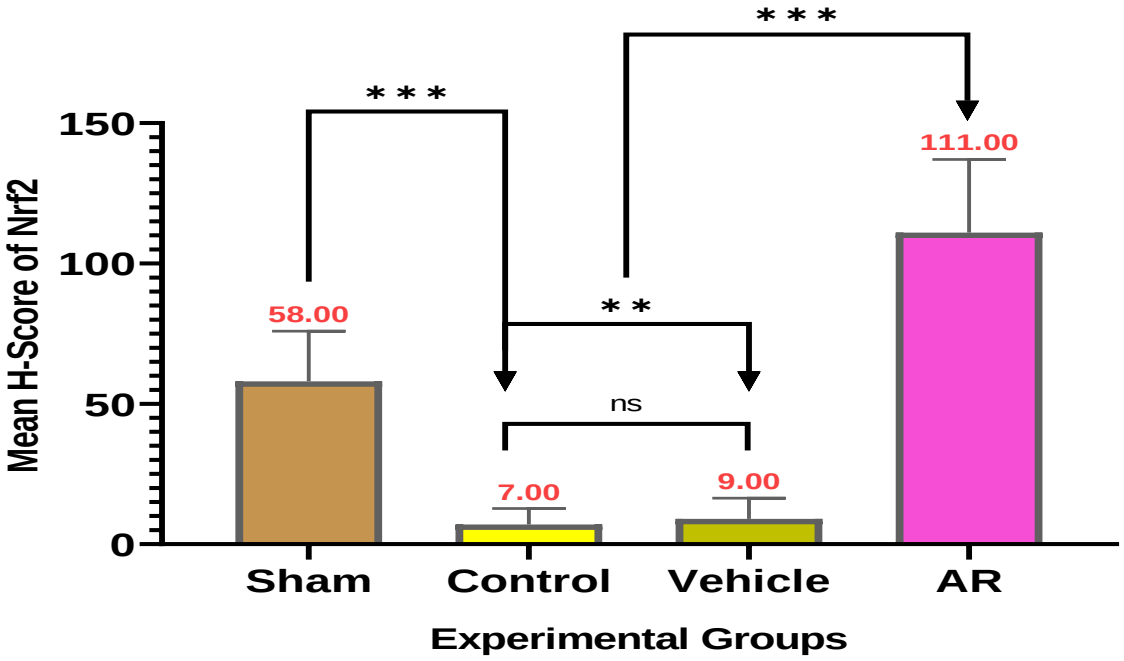

Figure (5): Represents the statistical analysis of Nrf2 expression in renal tissue of the four experimental groups (No of animals = 5 in each group).

Sham group vs. vehicle & control groups, ***P.value < 0.001
AR vs. vehicle & control groups, ***P.value < 0.001

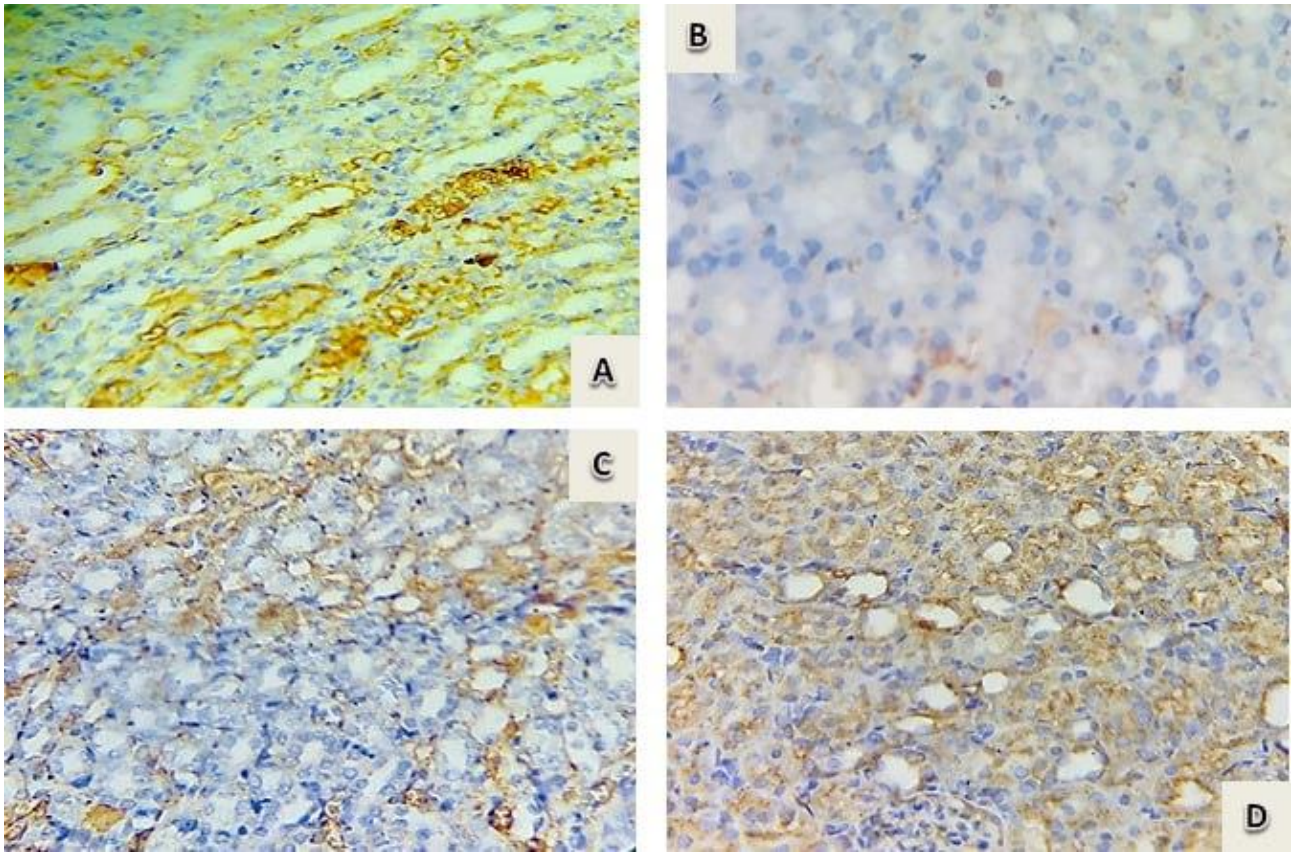

Figure (6): A) A cross section of left kidney represented a moderate positive cytoplasmic brown stain of Nrf2. × 400. Sham group. B) A cross section of left kidney showed a negative cytoplasmic brown stain of Nrf2. × 400. Control group. C) A cross section of left kidney appeared a slightly positive cytoplasmic brown stain of Nrf2. × 400. Vehicle group. D) A cross section of left kidney appeared a strong positive cytoplasmic brown stain of Nrf2. × 400. AR treated group.

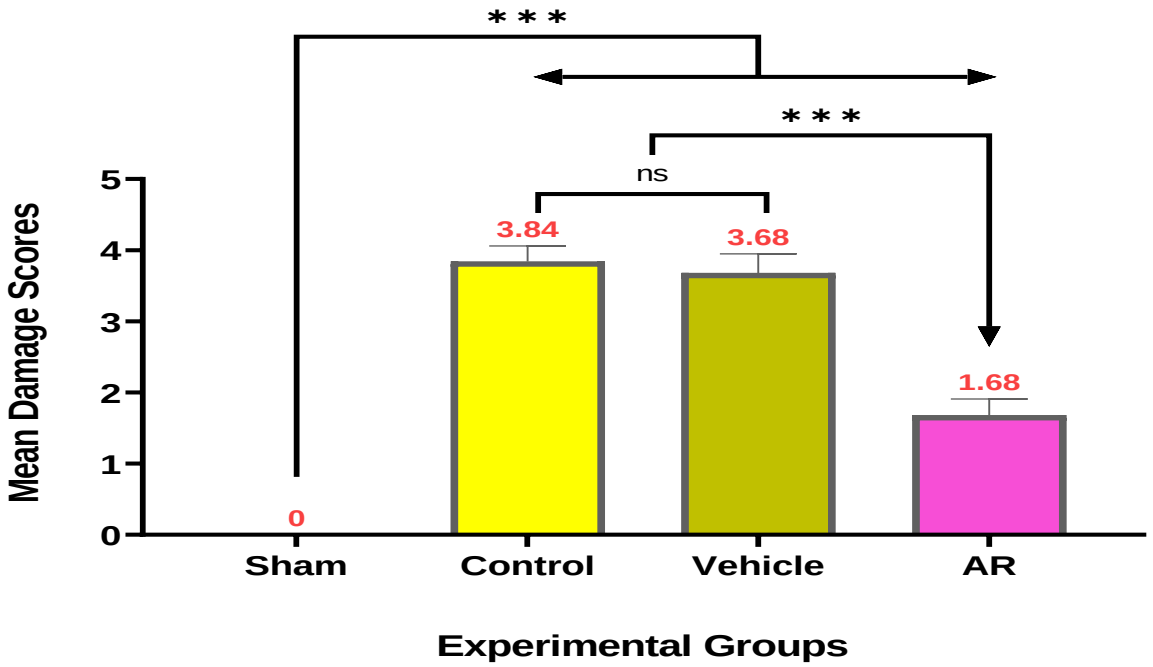

Figure (6): Represents the statistical analysis of damage score of renal structure of the four experimental groups (No of animals = 5 in each group).

Sham group vs. vehicle & control groups, ***P.value < 0.001
AR vs. vehicle & control groups, ***P.value < 0.001

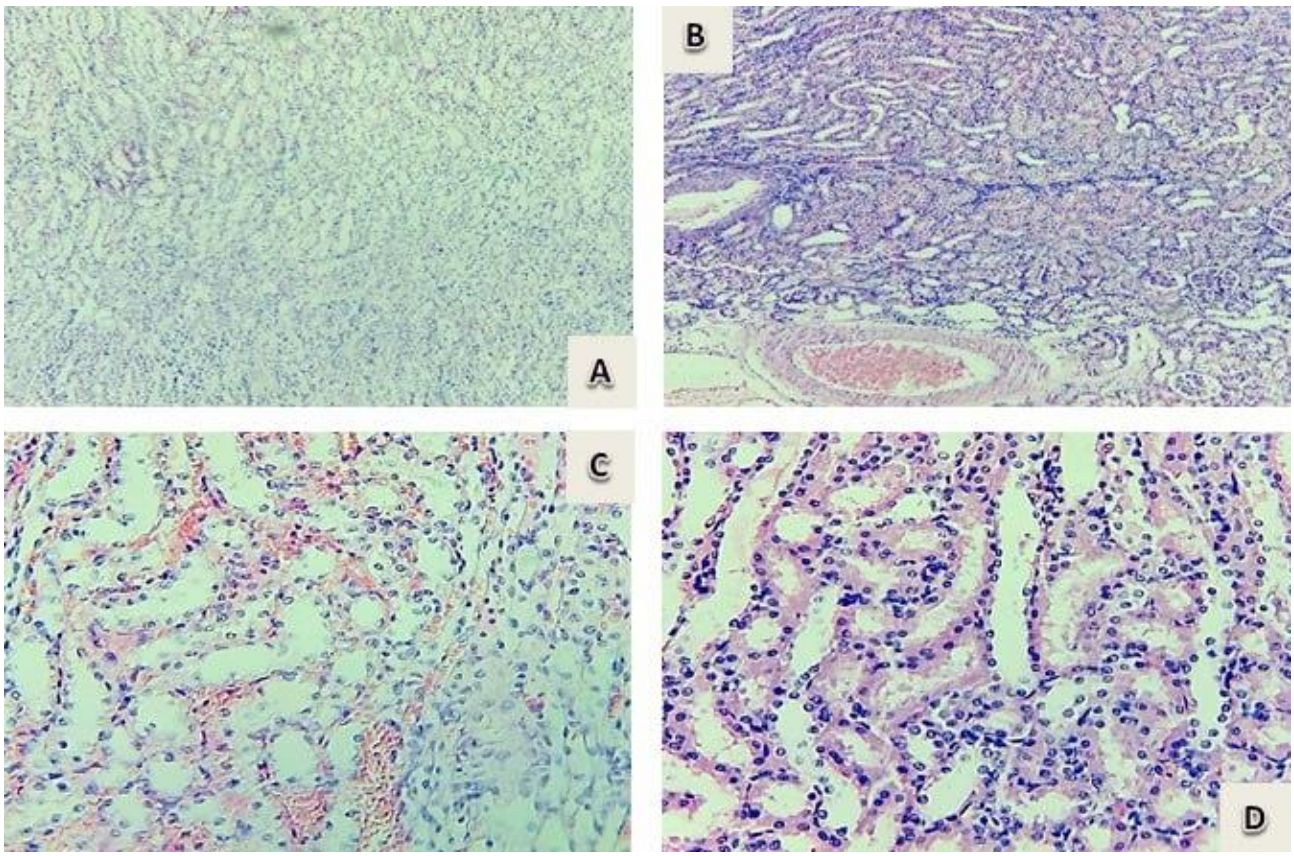

Figure (8): A) A microscopic cross section of left kidney represented normal tissues histology, normal renal tubules, normal cell size and there are no cast formations, cells edema or loss of brush boarder. Sham group. H & E stain × 400. B) A microscopic cross section of left kidney represented score 4 tissues modifications including severe cellular edema, cytoplasmic eosinophilia and strongly eosinophilic cast. Control group. H & E stain × 400. C) A microscopic cross section of left kidney represented score 4 tissues modifications including severe cellular edema, cytoplasmic eosinophilia and strongly eosinophilic cast. Vehicle group. H & E stain × 400. D) Microscopic cross section of left kidney represented

score 2 tissues modifications including few eosinophilic cast, moderate cellular edema and tubular dilatation. AR treatment group. H & E stain × 400.

Ethical Approval

All procedures involving the handling and experimentation on rats, as well as the conducted tests, were carried out in compliance with the applicable guidelines and regulations for the ethical use of animals \ Kufa University **(20547 in 29/8/2024).** The animals were housed in the animal facility at the College of Sciences, University of Kufa.

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