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The Anti-Apoptotic, Anti-Inflammatory And Anti-Oxidant Effects Of Olmesartan On Renal I/R Injury In Male Rat Model

Ahmed M. Abdul Hameed[1], Murooj L. Altemimi[1*], Rihab H. Al-Mudhafar[3], Dhefaf H. Al-Mudhafar[4], Najah R. Hadi[2]

[1]Department of Pharmacology and Therapeutics, Faculty of Medicine, Jabir IbnHayyan Medical University, Al Najaf Al-Ashraf, Iraq
[1*]Department of Pharmacology & Therapeutics, Faculty of Medicine, University of Kufa, Iraq
[2]Department of Pharmacology & Therapeutics, Faculty of Medicine, University of Kufa, Iraq,
[3&4]Middle Euphrates Unit for Cancer Researches, Faculty of Medicine, University of Kufa, Al Najaf Al-Ashraf, Iraq
Corresponding Author: Murooj L. Altemimi

ABSTRACT

Background: Ischemia reperfusion injury (IRI) is considered the main important factor that determined the morbidity and mortality in many disorders as example acute kidney injury (AKI), myocardial infarction (MI), ischemic stroke and sepsis. In certain situations as major surgery and organ transplantation, IRI is considered the main challenge that affected on healing and clinical outcomes. During ischemia, there is reduction in blood supply to ischemic organ and this will lead to poorly supply of oxygen (hypoxia) and nutrients and decrease the discharge of metabolic waste products and so will lead to accumulate carbon dioxide (CO2) and other debris. Severe ischemia and hypoxia for long time lead to micro-vascular changing in structure and dysfunction. When the ischemic tissue is reperfused by rapidly new blood that rich in oxygen and nutrient this will lead to micro vascular injury that is occur due to increase vascular permeability and leakage of fluid and protein from the capillaries to tissues lead to edema and damage. Also when the rapidly reperfusion occurred by highly oxygenated and nutrient blood to ischemic or damaging tissues lead to releasing of reactive oxygen species (ROS) from endothelial layer of capillaries and potentiate the inflammatory process and releasing of inflammatory mediators and factors such as tumor necrosis factor alpha (TNF-α), interferon gamma, interleukin 6 (IL-6), and interleukin-1β (IL-1β)and also releasing matrix metalloproteinase 9 enzyme (MMP9), complement protein and inflammatory or immunity cells as Neutrophil, macrophage, lymphocyte and others. Objective: This experimental research is done to investigate the possible Nephroprotective effect of Olmesartanon bilateral renal I/R injury in male rats through determining several renal function biomarkers as U & C, several inflammatory mediators as TNFα and MMP-9, antioxidant markers as TAC, several apoptotic markers as BCL-2 and BAX and also through histopathology scores. Materials and Method: In this study, 20 adult Wister Albino rats were used with 20-24 weeks in age and weighing 200-350 g. The adult rats were divided randomly into equal 4 groups (5 rats in each group) as the following:

1. Sham group: All 5 rats underwent the same anesthetic and surgical procedures for an identical period of time for ischemia and reperfusion without ischemia reperfusion induction. Renal tissues and blood samples were collected.
2. Control group: All 5 rats underwent median laparotomy under anesthesia, followed by 30min bilateral renal ischemia, and then renal tissues and blood samples were collected after 2h of reperfusion.
3. Vehicle group: All 5 rats pretreated with intraperitoneally injection of DMSO 30 min before ischemia reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours.
4. Olmesartan treated group: All 5 rats were pretreated with by intraperitoneally injection of Olmesartan 10 mg/kg 30 min before ischemia reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours.

Results: At the end of this experimental study, we showed that the serum level of U & C and tissue level of TNFα, MMP-9and BAX in sham group are significantly lowered than those in control and vehicle groups, also we showed that the serum level of U & C and tissue level of TNFα, MMP-9and BAX in OLM group is significantly lowered than those of control and vehicle groups. On the other hand, we showed that the tissue level of TAC and Bcl-2 in sham group are significantly higher than those in both control and vehicle groups. Also we showed that the tissue level of TAC and Bcl-2 in OLM group is significantly higher than those of both control and vehicle groups. In histopathology examination, we revealed that the OLM can significantly reduce the kidney injury and minimize the severity of tubular damage and deterioration in comparison with control and vehicle groups that are showed severe renal injury and tubular damage. Conclusion: From the overall results, we approved that the OLM is significantly reduce renal I/R injury in the bilateral renal I/R in adult male rats via their pleiotropic effects as anti-apoptotic, anti-oxidant and anti-inflammatory activities.

Keywords: Olmesartan, ischemia reperfusion injury, oxidative stress, Bcl-2, BAX, TNFα, MMP-9, inflammation, apoptosis & TAC

Correspondence:
Murooj L. Altemimi
Department of Pharmacology & Therapeutics, Faculty of Medicine, University of Kufa, Iraq

*Corresponding author: Murooj L. Altemimi

ABBREVIATIONS

OLM (Olmesartan), I/R (ischemia reperfusion), Bcl-2 (B-Cell Lymphoma-2), BAX (Bcl2 Associated X Protein), TNFα (tumor necrosis factor alpha), MMP-9 (matrix metalloproteinase-9), TAC (total anti-oxidant capacity), U & C (urea and creatinine), DMSO (dimethyl sulfoxide), ECM (extracellular matrix).

INTRODUCTION

Ischemia reperfusion injury (IRI) is the net result of an inflammatory process which is occurred when an organ suffering from a transient decrease or cessation of the blood flow, followed by restoration of perfusion [1]. IRI may induce in many clinical situations such as organ transplantation, vascular and cardiac surgery, shock, drug induced ischemia, and sepsis [2-3]. IRI consider one of the major challenges of organ transplantation, because it directly correlates to the graft rejection. 10% of early failures in transplantation are result of IRI. IRI is also accompanied with high rates of both acute and chronic grafts rejection [4]. During IR, tissue damage and/or death happen as a result of the firstly ischemic insult that is determined mainly by the severity and duration of blood flow interruption. Subsequently, generation of reactive oxygen species (ROS), because of re-oxygenation, starts events of IRI, result in a profound inflammatory response, apoptosis, and necrosis of irreversibly injured cells [5-6]. Inflammation and immune system play a serious function in the pathogenesis of renal IRI. The engagement of immune system is thought to cause the initial renal damage and mediate long term structural alteration including interstitial fibrosis or repair [7]. Inflammatory cells can potentiate kidney injury through production of mediators include cytokines, Chemokines, ROS, and eicosanoid; recruitment of leukocytes and up-regulation of adhesion molecules [8]. One important example about cytokines is TNFα. TNFα is considered one of major pro-inflammatory mediators or cytokines that mainly produced by activated macrophages, but it is also can be released from other innate and adaptive immunity cells such as Neutrophils, natural killer cells, T and B lymphocytes, mast cells and eosinophil[9].It is also formed and released from cells rather than immunity cells such as neurons, fat tissues, heart myocytes, endothelial cells, fibroblast and mesangial cells in glomeruli[10-12], [9]. TNFα can activate the macrophage and so this will lead to excrete other cytokines such as IL6, IL-1β and IL-8[13-14].TNFα also has an important role in growth controlling, cells and tissues differentiation and apoptosis and also cell cycle[10], [15-16].Other biological functions of TNFα inside human body are initiation of acute phase condition and stimulation of fever, activation of intracellular adhesion molecules, activation of macrophage and other phagocytic cells and so stimulates the phagocytosis and suppression or inhibition of appetite [10]. Also TNFα can be categorized as an anti-angiogenic and anti-neoplastic substance due to it can potentiate the innate and adaptive immunity system to destroy and kill the neoplasm cells [17-18]. MMPs are one of the inflammatory mediators that are structurally belong to zinc-related endopeptidase enzymes and are considered the major enzymes that responsible for cleaving the ECM components and also have an important role in remodeling of different organs and tissues during physiological and pathological situations [19-21]. MMP-9 (or Gelatinase-B) is considered one important type of MMPs family.MMP-9 can be produced and released from several types of cells and tissues such as, macrophages, endothelial cells, Neutrophils and fibroblasts [22]. It is formed as a pre-proenzyme that is a poly peptide composed from 19 amino acids with N-terminal region, then it is converted to proenzyme when it is released into extracellular matrix then it is undergone proteolytic activation either by other MMPs enzyme as MMP-3 or by other endogenous protease enzymes that can remove the pro-peptide area and produce the active MMP-9 [23-28]. MMP-9 has major roles in several physiological and pathological conditions. During some pathological situations such as the development of cancer, the degradation activity of MMP-9 to basement membrane will lead to support the cancer invasion and metastasis [29-31]. MMP-9 also has a role in angiogenesis and in the pathology of several fibrotic diseases such as kidney fibrosis, chronic kidney disease (CKD) and renal failure after kidney injury due to ischemia\reperfusion or due to diabetic nephropathy because MMP-9 is highly found in kidney cells and can remodel the parenchymal kidney tissues during those disorders[32-34]. Oxidative stress (OS) is an important pathway that participates in the pathogenesis of IRI through enhancing of reactive oxygen species (ROS) production [35]. ROS are highly reactive tiny molecules with potential harmful effect. They react with cellular component like lipids and proteins of cell membrane, carbohydrates, thiols and DNA leading to lipid peroxidation, inactivation of the enzyme, oxidation of glutathione, formation of organic radical, and destruction of the cell. However, ROS especially H_2O_2 can be beneficial for tissues mainly via their normal function in the cell signaling. Thus, the levels of ROS in a cell should be tightly regulated [36-37]. So, by using ROS scavengers and anti-oxidant agents to inhibit or block this pathological pathway or prevent the production of free radicals, is considered the major and most important strategy to prevent tissues injury during renal ischemia reperfusion and also protect them from damaging and death [38]. One of the final events that are occurred in ischemic injured parenchymal tissues of kidney is apoptosis. Apoptosis is considered a programmed cell death and a final destination of normal unwanted cells and pathological cells [39]. Apoptosis can be classified into physiologic apoptosis and pathologic apoptosis. Physiologic apoptosis can occur during normal conditions such as elimination of the harmful or injured cells or those cells that become aged or unusefulness. Also this type of apoptosis occurs through the normal development of the organs in which there is replacement of old and unwanted cells by new cells. In addition to that, physiologic apoptosis can occurs in highly hormone-sensitive and proliferative cells or tissues that are undergo rapid proliferation and differentiation in responses to several hormones such as growth factors and also ensuring that there are no or few inflammatory responses during the elimination of those cells. In immune cells, the physiologic apoptosis can eliminate the leukocytes after the finishing of immunity responses and prevent the occurrence of auto-immune disorders if these unwanted immune cells (leukocytes) remain without purge. On the other hand, the pathologic apoptosis can purge the unwanted sick cells that undergo severely DNA breakdown such as those are exposed to cytotoxic drugs, radiation, viral infection, cancer cells and also severe injury due to ischemia and hypoxia [40]. There are 2 major pathways of apoptosis and both of them depend on the activation of caspase enzymes. These pathways are **a)** mitochondrial or intrinsic pathway and **b)** death receptor or extrinsic pathway [39]**.** Bcl-2 is an anti-apoptotic protein that inhibits the different apoptotic stimuli through interaction with pro-apoptotic proteins BAX and BAK and improves the survival of the cells[41][42][43].The regulation or controlling of apoptosis is done by balancing the membrane of

mitochondria and stabilizing it and controlling its permeability and inhibits the releasing or leakage of death molecules such as cytochrome c[44].Bcl-2 also has other biological roles such as, it controls the dynamics of mitochondria, regulates the fusion of mitochondrial membrane, in beta cells of pancreas, Bcl-2 can control the insulin releasing and other metabolic activity [45]. The dysregulation or misbalancing of Bcl-2 level or activity or if there is defect or damaging in Bcl-2 gene, all these conditions will lead to imbalance among cell survival, division and death and finally lead to cause tumor growth especially in those tissues that are expressed high division activity such as breast, lung and prostate cancer, melanoma and chronic lymphocytic leukemia [46-48]. Bcl-2 is very important in the prevention of parenchymal kidney cells apoptosis during renal I\R and so it is considered a good biomarker that should be measured during this model of studies (renal I\R model in rat) to assess the severity of injury and to estimate the protection role of the treatment [49]. BAX is related to Bcl-2 family proteins and it is considered a pro-apoptotic endogenous agent that initiates the apoptosis during intrinsic pathway through increasing the permeability of mitochondrial membrane to release the cytochrome c and then stimulate the caspase cascade to elicit the apoptosis [50]. BAX has a pathological role in many renal diseases by stimulating the apoptosis and necrosis then cell death to lead to kidney fibrosis and failure in renal functions and the inhibition of BAX activity by anti-apoptotic agents can prevent these complications [51-55]. In normal condition, BAX found in the cytoplasm but when the apoptosis is stimulated, BAX will suffer from conformational change and transfer to bind to the membrane of organelles especially mitochondrial membrane [56-60]. This will lead to release the cytochrome c and several pro-apoptotic proteins from mitochondria, then the cytochrome c will activate the caspase-9 that is belong to intrinsic pathway of apoptosis. The active form of caspase-9 will stimulate the caspase-3 and the caspase-3 will potentiate the other caspase cascade to start the intrinsic apoptotic process [61]. Due to its crucial role in the induction of apoptosis during renal I\R injury and worsening other kidney diseases, BAX is considered a vital biomarker to assess the intensity of kidney parenchymal tissues injury and to estimate the protection and treatable effects of anti-apoptotic agents that are used in these conditions [62]. Olmesaratn (OLM) is one of the new ARBs agents that are acted selectively on AT1-receptor. AT1-recepter consider one and important receptor of AG II that is found in several parts of the body as in smooth muscle of the viscera and vasculature and has vasoconstriction, proliferation, inflammatory and apoptotic activity[63][64]. AGII considers one of the vasoactive peptide that coming from the activation of renin system found in kidneys[65][66].When AGII binds to AT1-R, it causes vasoconstriction of renal tubules, proliferation of cells and initiate the inflammatory process, in addition, it has non-hemodynamic action as potentiate the ROS formation and apoptosis so the blocking of this receptor (AT1-R) by ARBs prevent the bad action of AGII on renal system after IR and give protective effect by act as anti-inflammatory, anti-apoptotic, anti-proliferation and anti-oxidant and so prevents the damaging of renal tubules and cells [67-69]. The physiologic effects of Olmesartan include reduction in the blood pressure, decrease ADH and aldosterone concentration in the body, decrease the activity of cardiac muscle and elevate Na excretion from renal tubules. Olmesartan by its mechanism of action can block the negative compensatory mechanism of RAAS on several vital organs such as kidney, heart and blood vessels. So, Olmesartan can stop or prevent or decrease the progression, complications and pathogenesis of several cardiovascular diseases (CVDs) such as hypertension, HF and IHD and also several kidney disorders such as AKI due to I\R injury and diabetic nephropathy. Particularly, in AKI due to I\R injury, Olmesartan can prevent the complications of this condition via act as anti-oxidant to stop the adverse effects of ROS, as anti-inflammation to block the pathological role of cytokines and Chemokines during renal I\R and as anti-apoptotic and anti-proliferation to inhibit the apoptosis and cell death during ischemia and hypoxia of renal cells [70-72].

MATERIAL AND METHOD

Site and ethical consideration of the research

The study was done in the department of pharmacology and therapeutic and 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 Medicine. Whole procedures were done according to the recommendations of the Committee.

Animal grouping

In this study, 20 adult Wister Albino rats were used with 20-24 weeks in age and weighing 200-350 g was getting from the Center of Control and Pharmaceutical research/Ministry of health. Animals were harbored in animal house of Faculty of Science/ University of Kufa with a temperature controlled 20-25ºC and 60-65% humidity with a fitted 12 hrs light and 12 hrs dark cycle for 14 days before start of the procedures. In addition, the rats were freely access to food and water. In this study, the rats were divided randomly into 4 equal groups, 5 rats in each group and as the following:

1. **Sham group:** All 5 rats underwent the same anesthetic and surgical procedures for an identical period of time for ischemia and reperfusion without ischemia reperfusion induction. Renal tissues and blood samples were collected.
2. **Control group:** All 5 rats underwent median laparotomy under anesthesia, followed by 30min bilateral renal ischemia, and then renal tissues and blood samples were collected after 2h of reperfusion [73-75].
3. **Vehicle group:** All 5 rats pretreated with intraperitoneally injection of DMSO 30 min before ischemia reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours [76].
4. **Olmesartan treated group:** All 5 rats were pretreated with by intraperitoneally injection of Olmesartan 10 mg/kg 30 min before ischemia reperfusion injury and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours [77].

Renal ischemia reperfusion injury rat model

All the rats were anesthetized by intraperitoneal injection 100 mg/kg ketamine hydrochloride and 10mg/kg of xylazine hydrochloride. The animals were placed on a heat plate to preserve the rat body temperature at about 37 ºC. Hair of the abdominal area was shaved and wiping with antiseptic to avoid infection followed by making midline incision by cutting the abdominal skin and then the abdominal muscle to expose the renal pedicles. Clamping of left and right renal pedicles for half hour with

un 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. After the reperfusion time (2 hrs.) open the suture and about 3 ml of blood were draw from the heart, followed by bilateral nephrectomy that washed with precooled phosphate buffer saline (PBS) to cleaning the kidney from blood. Finally sacrificed the rat by heart puncture [78], the left kidney was cut sagittal into 2 halves. The first half was kept in deep freeze for bimolecular assessment. While the second half was inserted in 10% formalin then embedded in paraffin for histopathological and immunohistochemical assessment.

Preparation of the drug

The drug was prepared immediately before using by dissolved in DMSO as descripted by manufacturer (Medchemexpress).

Assessment of kidney function

The drawn blood was put in a gel tube without anticoagulation and left for about 30 min. at room temperature then centrifuged at 4000 rpm for 15 min. to obtained serum which used within 3 hrs to measure the level of serum urea (U) and creatinine (C) by commercially available assay kits.

Assessment of tissue TNFα, MMP-9 and TAC

The frozen kidney portion was divided into small fragments and washed with cold PBS then the tissue was weighted and firstly homogenized by mortar and pestle with 1:10 (W/V) 0.1 M of precooled PBS (PH 7.4) contain 1% of protease inhibitor cocktail and 1% Triton 100X [79][80]. For good homogenization, further breakdown the cell membranes achieved by subjected the homogenate to high intensity ultrasonic liquid processor. Lastly the homogenate was centrifuged at 10000 rpm for 10 min. at 4 ºC. The supernatant was utilized to determine the level of TNFα, MMP-9 and TAC by ELISA kit.

Histopathological analysis

Half left kidney was dehydrated and cleared then embedded in paraffin and cut to sections of 5µm thickness by rotary microtome. Thereafter fixing the tissue section on slides, stained with hematoxylin eosin dye, and fixed by cover slid to prepared for examination by microscope. Renal tissue damage was evaluated by two experienced pathologists in a blind way taking in consideration 6 randomly selected fields. The sections were classified with a scale design for assessment the degree of renal injury like swelling of renal epithelial cell, desquamation of epithelial cells into the lumen, eosinophilic cast formation, loss of brush border, inflammatory reaction, degeneration of vascular and tubular necrosis. The score system that was used formed from five scores: score 0 for normal kidney tissue, score 1for kidney damage area less than 25%, score 2 for kidney damage area range from 25%-50%, score 3 for kidney damage area 50%-75%, score 4 for kidney damage area more than 75% [81].

Immunohistochemistry assessment

Immunohistochemistry was performed to assess Bcl-2 and BAX in kidney tissue. 5µm paraffin embedded sections were stained by utilizing immunostaining procedure. Briefly, sections were subject to deparaffanized, rehydration, antigen repairing by exposed to retrieval buffer, and inhibiting endogenous peroxidase activity by 3% H_2O_2. The sections were incubated with Bcl-2 or BAX polyclonal antibody (1:200, bioassay) overnight at 4 ºC. After washing, the slices incubated for 1 hr. with conjugated secondary antibody, washed and subjected to horseradish peroxidase for half hour. After that the sections incubated with fresh 3, 3′-diaminobenzidine for 8 minutes. Finally, hematoxylin stain was used for counter stain. Then observe the staining under the microscope. The protein expression of Bcl-2 or BAX was calculated by H-score method (ranged 0-300) that resulting from multiplying the intensity and percent of the staining area. The intensity of stain was scored as 0-3, 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percent of cells stained was graded from 0- 100% [82].

Statistical analysis

Statistical analyses were performed using SPSS 26.0 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 and the IHC-P, the Kruskal-Wallis test was used to assess the statistical significance of difference across multiple groups in total severity score (mean score) for histopathological renal changes and mean H. score for IHC-P. In all tests, $P \leq 0.05$ was considered statistically significant.

RESULTS

Olmesartanimprove renal function

Rats in control and vehicle groups exhibited a significant increase in serum U and C in comparison with sham group. Olmesartan pretreatment group was significantly reduced the two markers of kidney function comparing with control and vehicle groups (Figure 1 and 2).

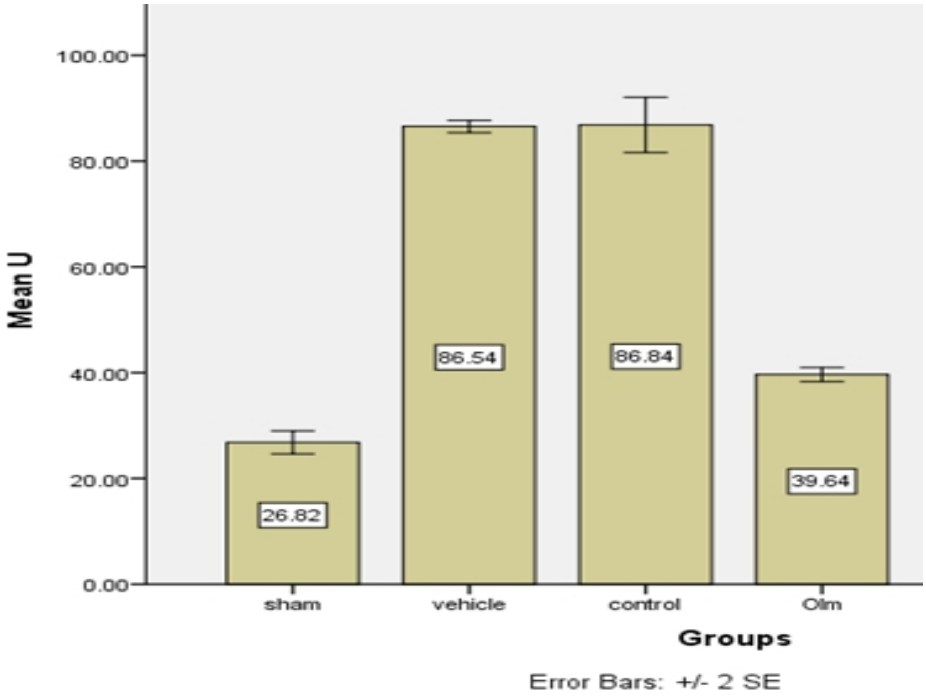


Figure (1): Mean serum level of U (mg/dl) of the four experimental groups at the end of the study (No of animals = 5 in each group)

Sham group vs. vehicle & control groups, P. value = 0.00001

OLM vs. vehicle & control groups, P. value = 0.00001

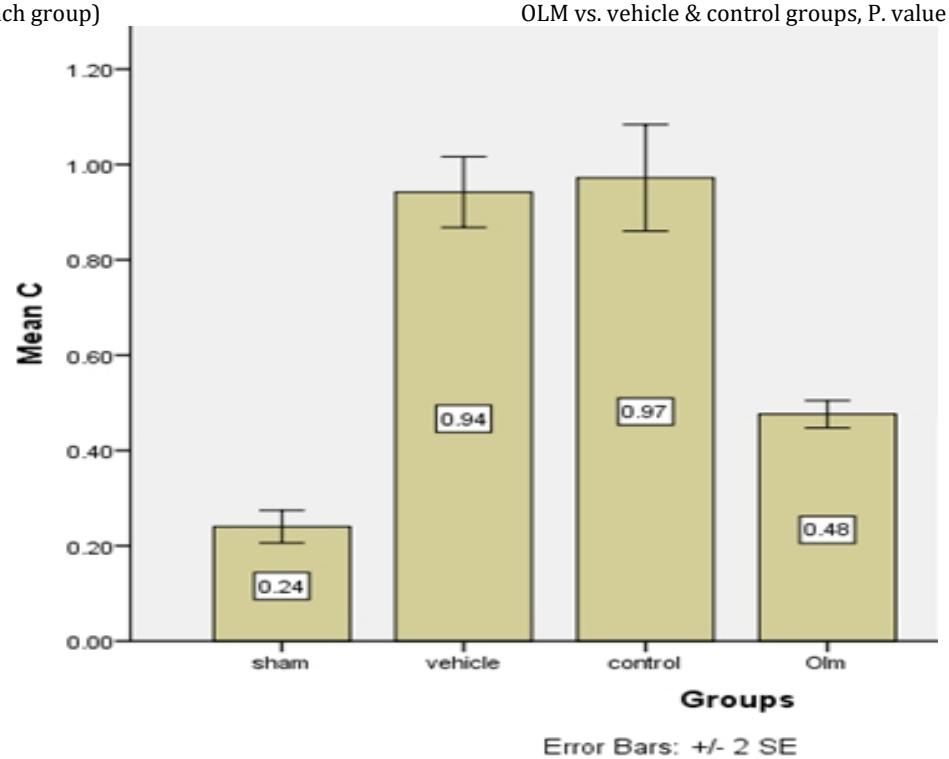


Figure (2): Mean serum level of C (mg/dl) of the four experimental groups at the end of the study (No of animals = 5 in each group)

Sham group vs. vehicle & control groups, P. value = 0.00001

OLM vs. vehicle & control groups, P. value = 0.00001

Olmesartan attenuated oxidative stress and improve TAC in renal tissue

In our experimental research, we showed that the renal tissue level of TAC in sham group was significantly ($p < 0.05$) higher than those level in both control and vehicle groups. The renal tissue level of TAC of OLM pretreated group was significantly ($p < 0.05$) higher than those level in both control and vehicle groups (figure 3).

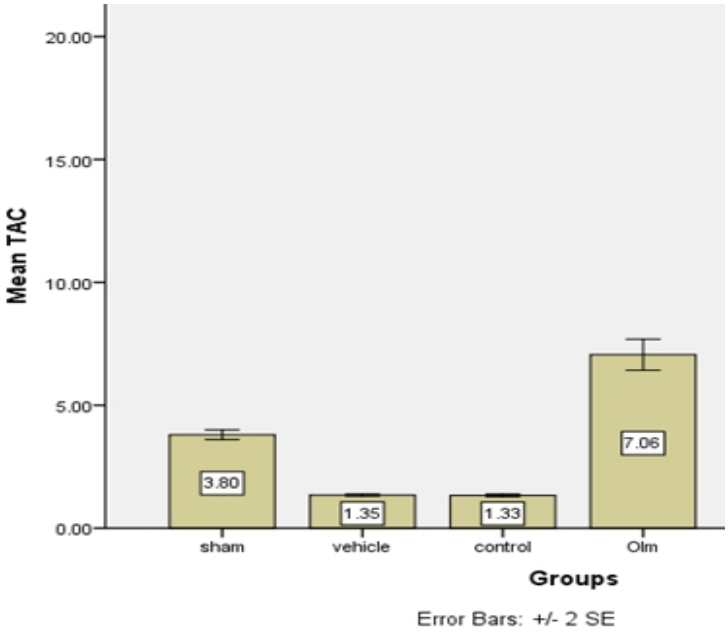


Figure (3): Mean renal tissue level of TAC (U/ml) of the four experimental groups at the end of the study (No of animals = 5 in each group)
Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001

Olmesartan decreased the inflammatory markers in renal tissue (TNFα and MMP-9)

Protein expression of the inflammatory mediators, TNFα and MMP-9, were increased significantly in kidney homogenate of control and vehicle rats in comparison with sham rats. 10 mg/kg of OLM significantly diminished the expression of TNFα and MMP-9in comparison with control and vehicle rats (figure 4 and 5).

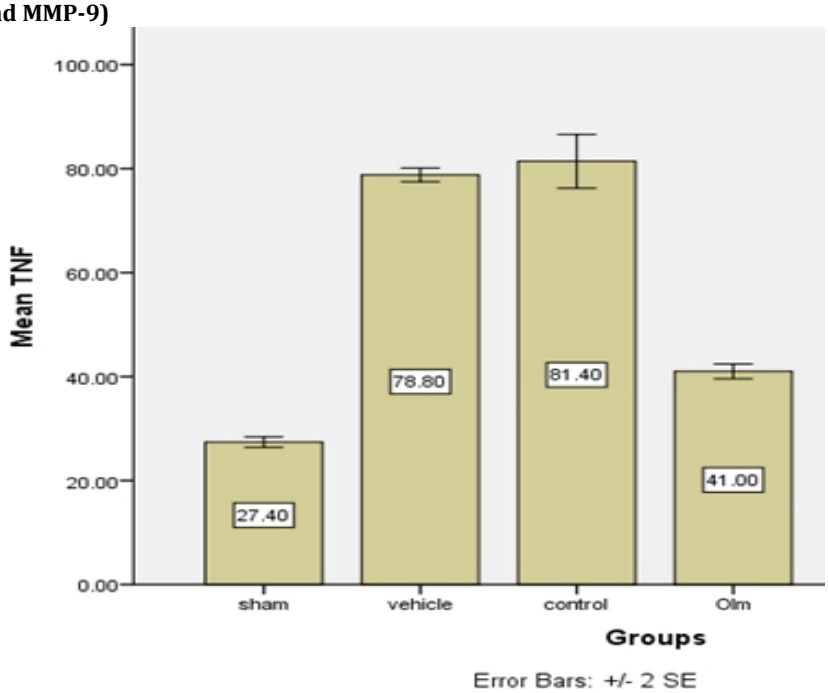


Figure (4): Mean renal tissue of TNFα (ng/ml) of the four experimental groups at the end of the study (No of animals = 5 in each group)

Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001

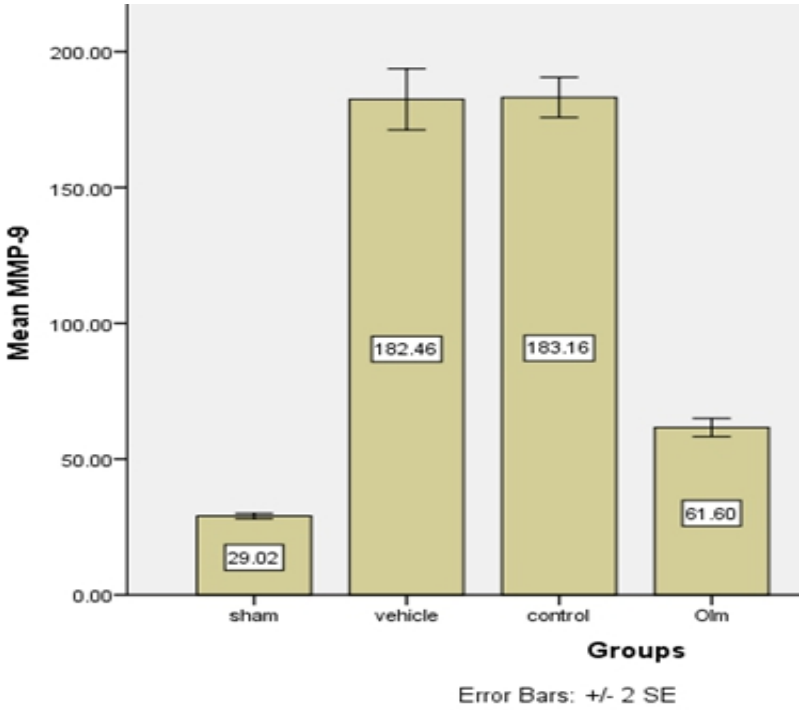


Figure (5): Mean renal tissue level of MMP-9 (ng/ml) of the four experimental groups at the end of the study (No of animals = 5 in each group)
Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001

Olmesartan upregulated Bcl-2 expression

In this experimental study, we approved that the expression of Bcl-2 in renal tissue in sham group was significantly ($p < 0.05$) higher than (low H. score mean) that the expression in both control and vehicle groups (high H. score mean). The renal tissue level of Bcl-2 of OLM pretreated group was significantly ($p < 0.05$) higher than those levels in both control and vehicle groups (Figure 6 and 7).

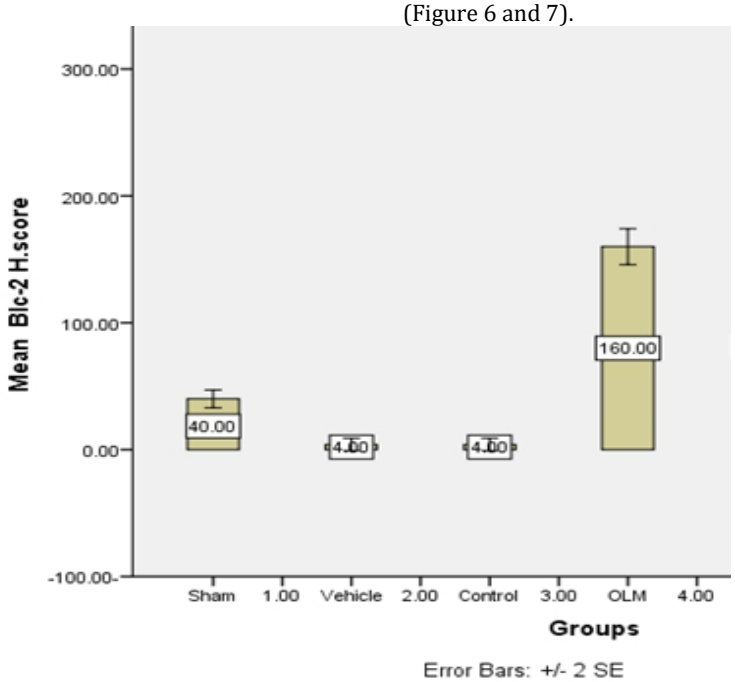
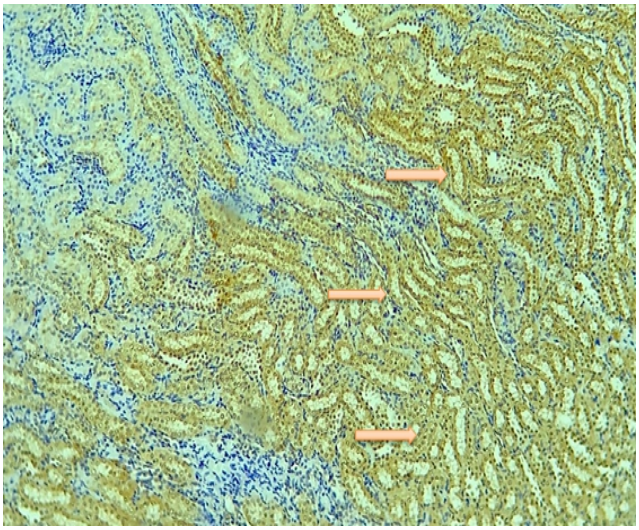
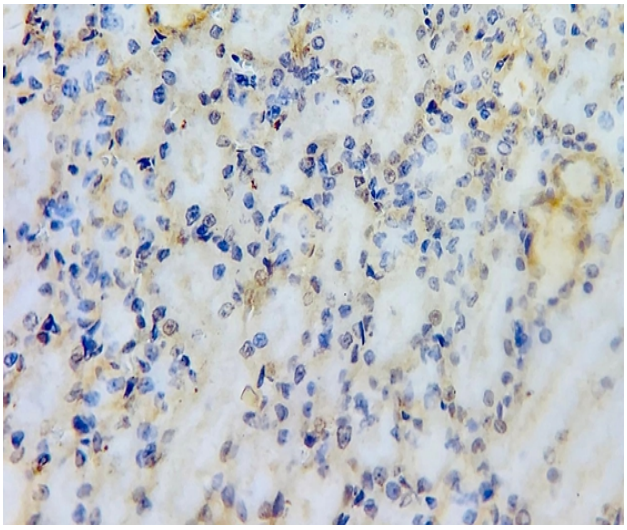


Figure (6): Mean H. scores of Bcl-2 in renal tissue of the four experimental groups at the end of the study (No of animals = 5 in each group)

Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001

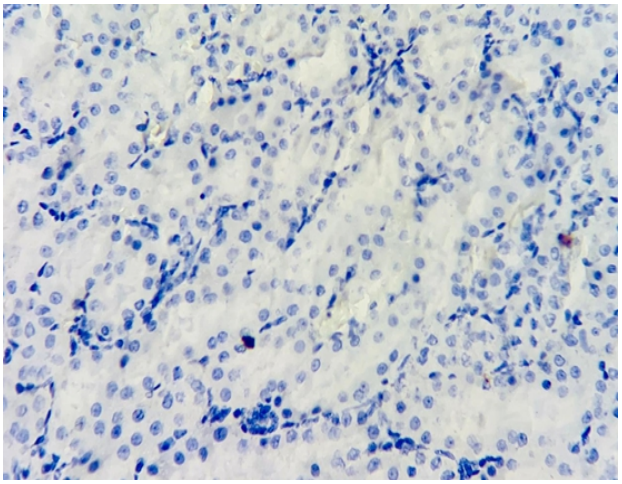


B.Bcl-2 control group, negative stain × 400



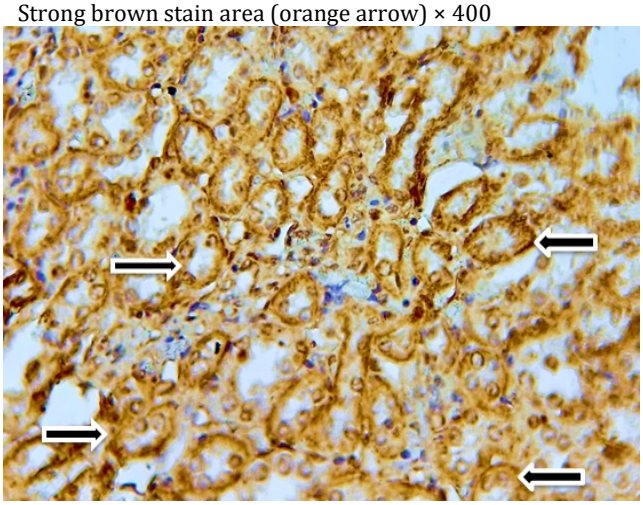
A. Bcl-2 sham group

Strong brown stain area (orange arrow) × 400



C. Bcl-2 vehicle group, negative stain × 400**D.** Bcl-2 expression of OLM pretreated group Strong brown stain (black arrow) × 400

Figure (7): Immunohistochemical staining for Bcl-2



Olmesartan down regulated BAX expression

In this experimental study, we approved that the expression of BAX in renal tissue in sham group was significantly ($p < 0.05$) lower than (low H. score mean) that the expression in both control and vehicle groups (high H. score mean). The renal tissue level of BAX of OLM pretreated group was significantly ($p < 0.05$) lower than those levels in both control and vehicle groups (figure 8 and 9).

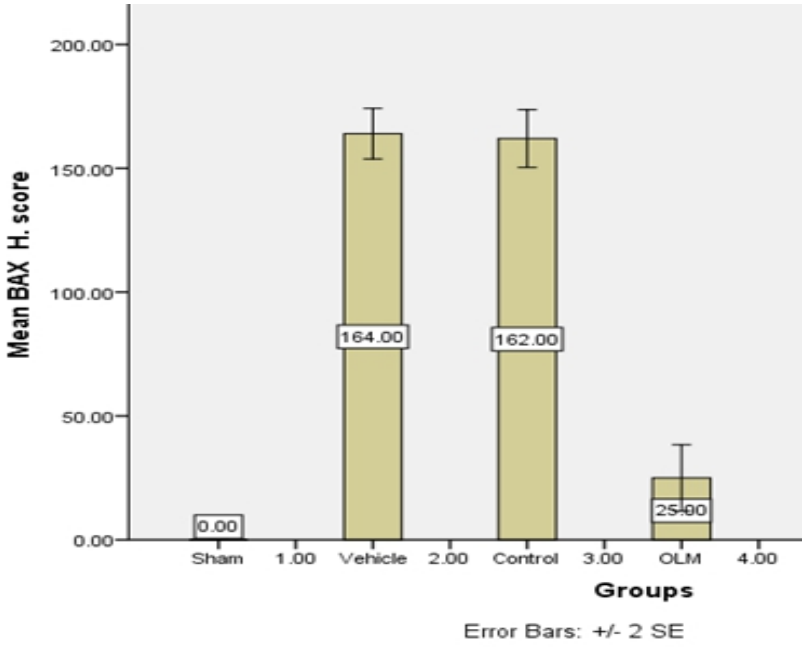
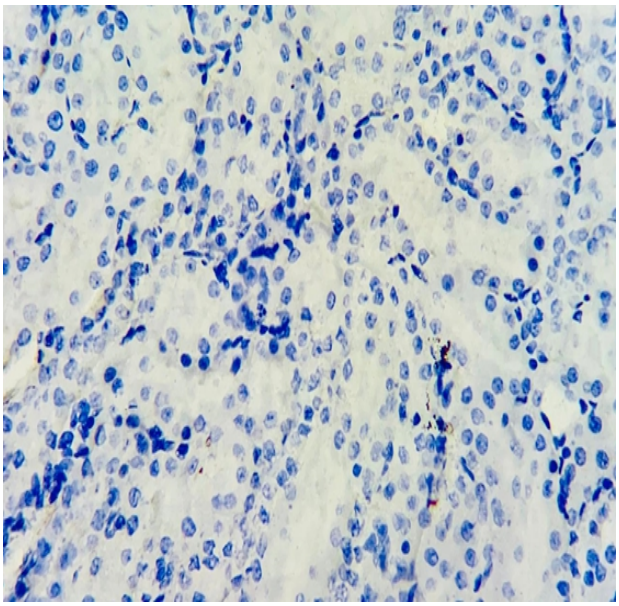
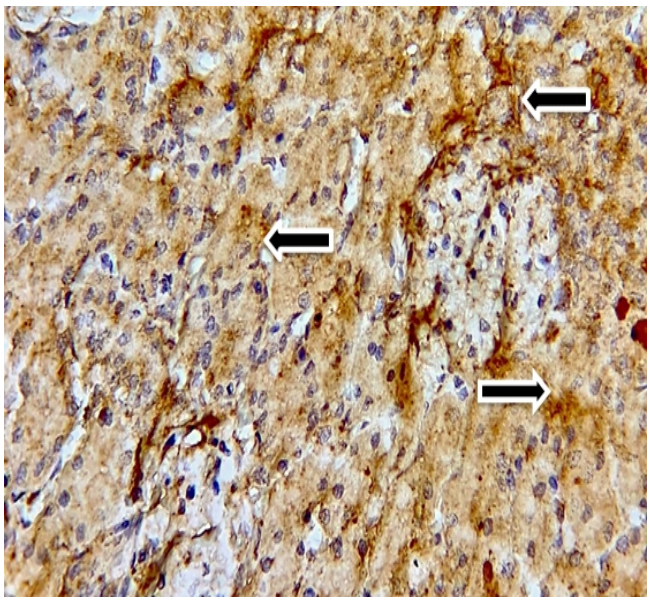


Figure (8): Mean H. scores of BAX in renal tissue of the four experimental groups at the end of the study (No of animals = 5 in each group)

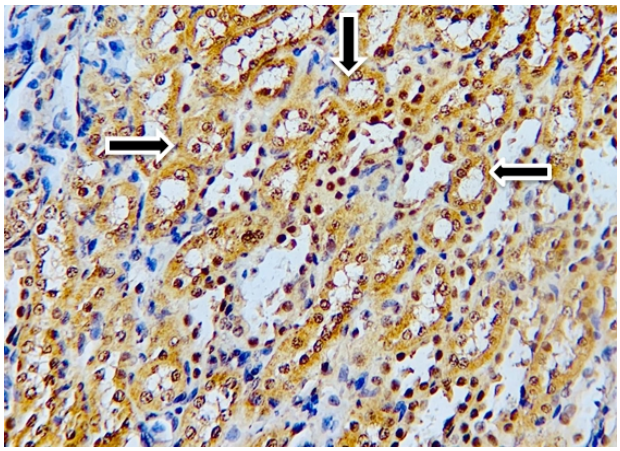
Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001

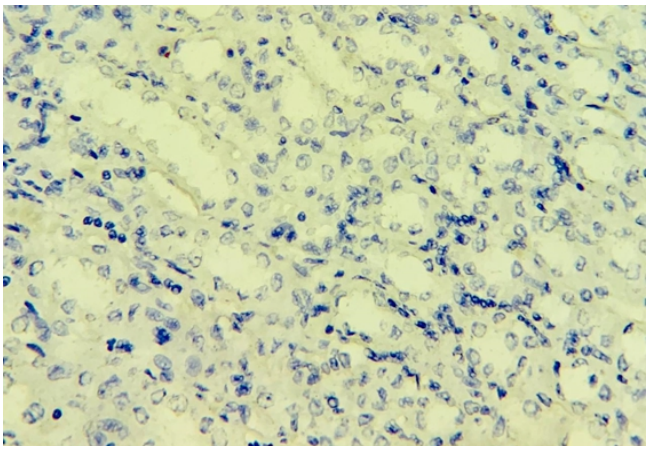


A. BAX sham group, negative stain × 400



B. BAX control group Strong brown stain (black arrow) × 400





C. BAX vehicle group, **D.** BAX expression in OLM pretreated group
Strong brown stain (black arrow) × 400 Negative stain × 400
Figure (9): Immunohistochemical staining for BAX

Olmesartan minimized kidney injury

Histopathological examination showed no renal injury in the sham group. In control and vehicle groups, an increased number of damaged tubules and cell dilatation were noticed in comparison with the sham group (P< 0.05). Olmesartan pretreated group showed little histological change in contrast to the control and vehicle groups (P< 0.05) (figure 10 and 11).

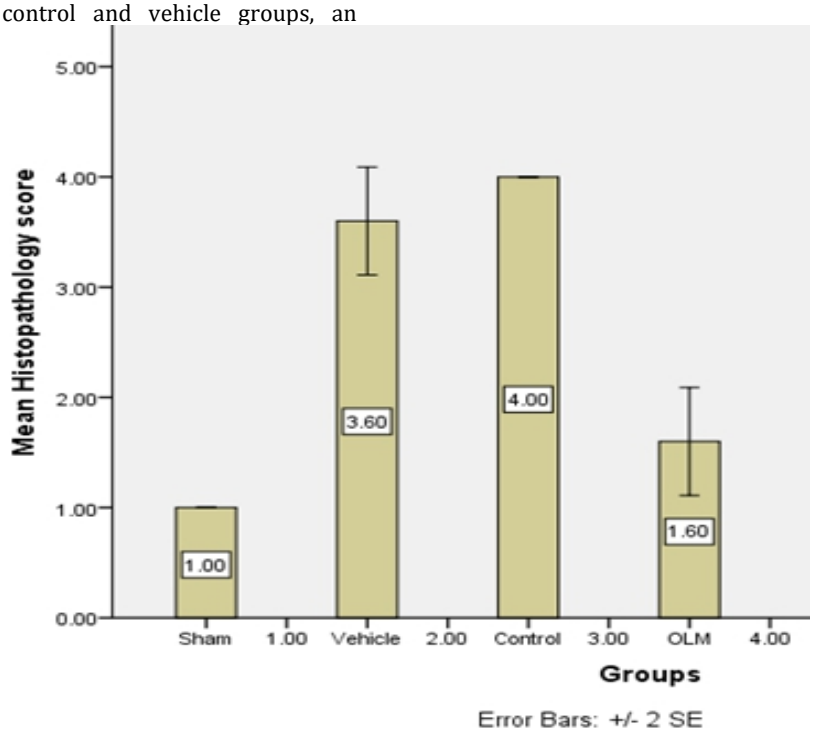
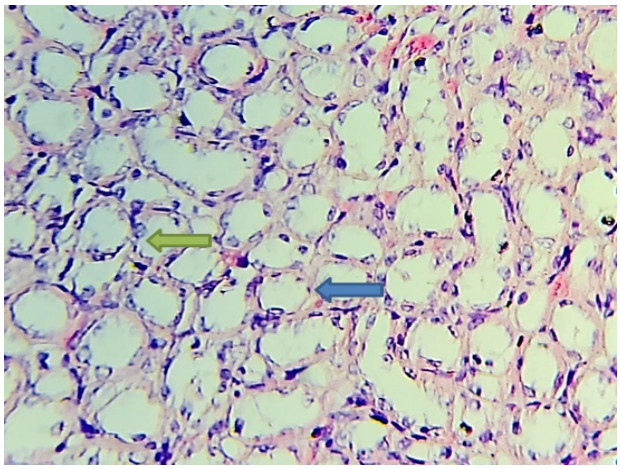
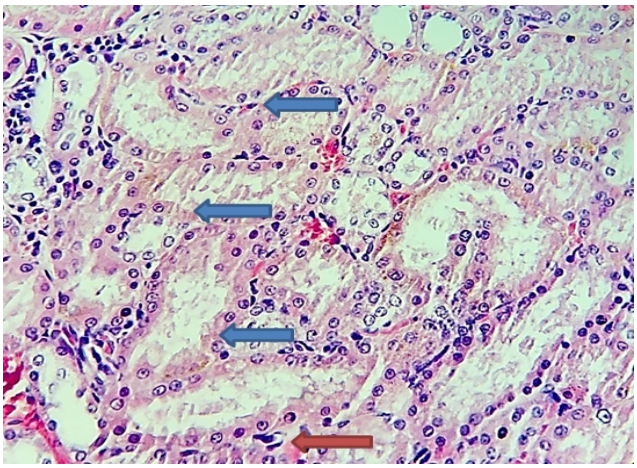


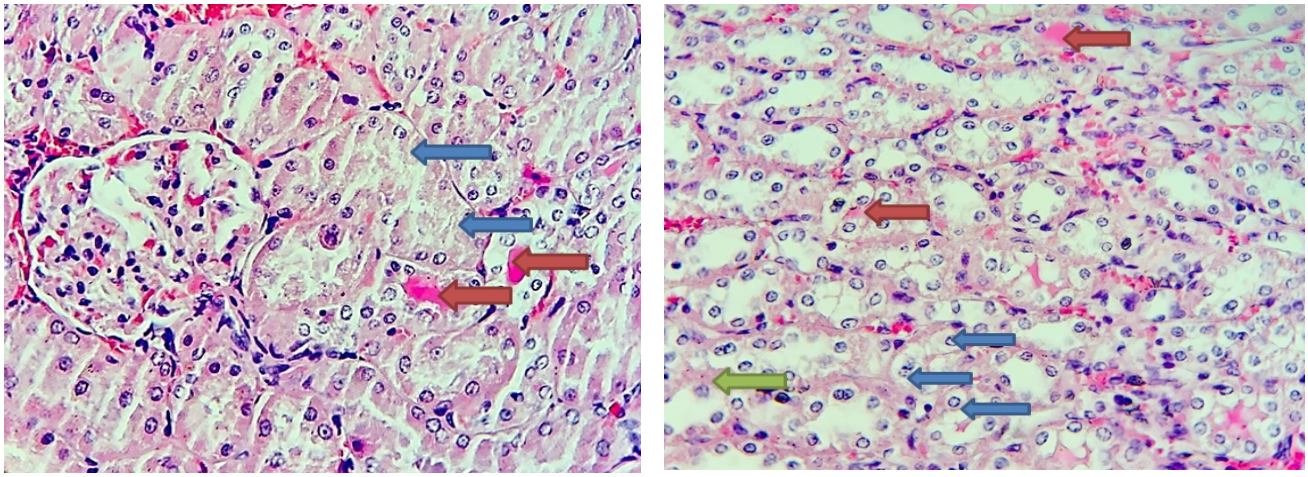
Figure (10): Score severity mean of renal tissue histopathology of the four experimental groups at the end of the study (No of animals = 5 in each group)
Sham group vs. vehicle & control groups, P. value = 0.00001
OLM vs. vehicle & control groups, P. value = 0.00001



A. Photomicrograph of the left kidney **B.** Photomicrograph of the left kidney section in section in sham group: Shows normal histology control group:



Shows score 4 ischemic changes of renal tubules (blue arrow) and normal cell including cellular swelling and cytoplasmic size (green arrow) x400eosinophilia(blue arrows) and eosinophilic cast (red arrow) x 400



C. Photomicrograph of the left kidney section in **D.** Photomicrograph of the left kidney section vehicle group: Shows score 4 ischemic changes in OLM pretreated group: Shows score 1 **including** cellular swelling and cytoplasmic ischemic changes including eosinophilic cast eosinophilic (blue arrows) and eosinophilic cast (red arrows), cellular swelling and tubular (red arrow) x 400 dilatation (blue arrows) and cytoplasmic Eosinophilic (green arrow)
Figure (11): H & E staining of left kidney section for histopathological examination

DISCUSSION

IRI is considered one of the most important factors that has a major role in morbidity and mortality of many diseases such as AKI, ischemic stroke, MI and sepsis. Also IRI is considered a major challenge in some conditions such as organ transplantation and major surgery which can affect on healing and clinical outcomes. Through ischemia, the blood supply to the vital organs will decrease and can cause hypoxia (low O2 concentration) and also decrease in nutrients supply and at the same time, the debris and CO2 will accumulate. Long duration of ischemia and hypoxia will lead to structural changes and dysfunction in micro-blood vessels. During rapid reperfusion to the ischemic organ, high amount of blood will flow and lead to cause series of complications in that organ. Inflammation process is one of those problems that can irritate the tissues and lead to farther cascades of complications, ROS formation and initiate apoptosis [83-84]. So in our thesis, we estimated the Nephroprotective effect of Olmesartan, against control renal IRI experimentally.

Effect of Olmesartan on renal function parameters (Urea and Creatinine)

This experimental research revealed that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction is significantly ($P<0.05$) decreasing the levels of Urea and Creatinine in comparison with those levels in both control and vehicle groups. This result means that the Olmesartan has a preservative effect on renal function parameters (Urea and Creatinine) after renal IRI induction on rat model. This finding is in agreement with other studies. Habibi J, et al., 2019 and Eltzschig HK, and Eckle T, 2011 studies showed that the treatment group with selective AT-1 inhibitors as Valsartan, Olmesartan or others will diminished the levels of Urea and Creatinine and so can conserve the normal renal function parameters[67-85].

Effect of Olmesartan on renal parenchyma

This experimental study appeared that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction can significantly ($P<0.05$) decrease the severity of kidney injury in comparison with the severity of renal injury in both control and vehicle groups. The mean of total severity score in this pretreatment group confirmed a mild to moderate kidney injury. Our result is in agreement with other researches. Jing W, et al., 2017 and Vaziri N D, et al., 2007 studies showed that the pretreatment group with selective AT-1 receptor blocker Olmesartan or other one had statistically significant histopathological lack of brush border, tubular vacuolization and tubular dilatation [86-87].

Effect of Olmesartan on the inflammatory mediators (TNFα)

This experimental study confirmed that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction can significantly ($P<0.05$) lower the level of inflammatory cytokine (TNFα) in ischemic tissues of kidney in comparison with the levels of this inflammatory cytokine in both control and vehicle groups. This result means that the Olmesartan has a Nephroprotective effect in renal tissues that are undergone ischemia then reperfusion. Our result is in agreement with other researches. One experimental study revealed that the Doxorubicin cytotoxic agent can significantly elevate several inflammatory mediators as TNF-α, ICAM-1, and IL-1β and also increased the expression of NF-κB inflammatory pathway. On the other hand, L-carnitine and Olmesartan, especially as a combination formula, can significantly decreased the expression of NF-κB pathway, and so suppressed the level of several inflammatory cytokines as TNF-α and IL-1β, so the pretreatment by this combination can provide a better anti-inflammatory effect [88]. Kim JM, et al., 2011 research revealed that the inhibition of AT-1 receptors by selective ARBs can suppress the activity of NF-κB pathway and so act as anti-inflammatory agents [89].

Effect of Olmesartan on Matrix Metalloproteinase 9 (MMP 9)

This experimental research confirmed that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction can significantly ($P<0.05$) lower the level of Matrix Metalloproteinase 9 (MMP 9), which is also one of the inflammatory mediators, in injured tissues of kidney in comparison with the levels of this inflammatory marker in both control and vehicle groups. This result also reveals that the Olmesartan has a

Nephroprotective effect in renal tissues that are undergone ischemia then reperfusion. When the expression of TNFα and IL-1β are increased in inflamed or injured tissues, this will lead to elevate the level of MMPs enzyme such as MMP-9 and MMP-2[90][91]. Our finding is in settlement with other researches. One experimental study showed that the tissue staining for MMP-9 and MMP-2 was decreased in rats that are suffering from intestinal mucositis when pretreated with Olmesartan 5mg/kg so this will lead to alleviate the intestinal mucosa proteolysis and reduce the risk of inflammation and ulceration in intestinal tissues [92].

Effect of Olmesartan on Total Antioxidant Capacity (TAC), oxidative stress and ROS formation

This experimental research showed that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction can significantly ($P<0.05$) increase the level of TAC, which is considered one of the normal antioxidant mechanism in the body, and at the same time decrease the oxidative stress and free radical production in ischemic tissues of kidney in comparison with those in both control and vehicle groups. This result reveals that the Olmesartan has an antioxidant effect in injured and inflamed renal tissues that are undergone ischemia then reperfusion. Our result is in agreement with other studies. One experimental research on rats showed that the induction of colitis by Acetic Acid (AA) will lead to potentiate the oxidative stress condition and this can be indicated through increasing in the levels of MPO and Malondialdehyde (MDA), which is also one type of ROS, and on the other hand, reducing the levels of super oxide dismutase (SOD), TAC, catalase (CAT) and glutathione (GSH). This study confirmed that the Olmesartan can act as antioxidant agent by blocking the AT-1 receptor and so reduces the MPO and MDA levels and activates the antioxidant mechanism through enhancing the TAC, SOD and other free radicals scavengers. So it can prevent or alleviate the oxidative damage and potentiate healing in inflamed and injured colonic tissues [93].

Effect of Olmesartan on apoptotic markers (Bcl-2 and BAX)

This experimental research confirmed that the pretreatment with selective AT-1 receptor blocker Olmesartan before ischemia induction can significantly ($P<0.05$) modulates the ratio of BAX/Bcl-2 in injured renal tissues in comparison with those in both control and vehicle groups. The mechanism of prevention the apoptosis by ARBs is through the upregulated the expression of Bcl-2 gene and down regulated the expression of BAX gene. Bcl-2 protein is considered as an anti-apoptotic endogenous mediator while BAX protein is considered as an apoptotic initiation mediator [94]. So by increasing the expression of Bcl-2 and at the same time decreasing the expression of BAX, ARBs can stabilize the permeability of mitochondrial membrane and prevent cell death [95-96]. Our finding is in settlement with other studies. Wang R, et al., 1999 showed that the Olmesartan can down regulate the expression of BAX/Bcl-2 ratio so prevent the initiation of apoptosis and cell death [97]. Other experimental study result on rat model showed that the Nephroprotective effect of ARBs is mediated by the up regulation of Bcl-2 level and decreasing of BAX and caspase-3 expression [98].

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