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Modulation Renal Expression of mTOR Gene via Calcitriol on RIRI in Rats

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ABSTRACT

Background: It seems that IRI is primary factor causing morbidity and death in a number of conditions; including sepsis, MI, and AKI. The microvascular system undergoes structural and functional alterations as a result of ischaemia. Additionally, endothelial layer of capillaries produces ROS in response to the fast reperfusion of ischaemic tissues by blood, which exacerbates the NFκB pathway and inflammatory process. Several inflammatory components such as IL-1β, and TNF-α are released.

Objective: This research is done to examine the effectiveness of Calcitriol in attenuating renal injury during IR through modulation mTOR gene.

Method: 28 Wister Albino rats were randomly assigned to four equal groups, (N=7):

Sham: Rats undergone laparotomy 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.

Calcitriol: Rats received an intraperitoneal injection of Calcitriol three days prior to RIRI.

Results: TNFα, IL-1β, F2 Isoprostane, BAX, and KIM-1 tissue levels in sham were significantly lower than in vehicle and control. Additionally, the findings showed that compared to the vehicle and control, the Calcitriol had significantly decreased levels of KIM-1, TNFα, IL-1β, F2 Isoprostane, and BAX. It also showed that tissue content of Bcl2 and mTOR was significantly greater in Calcitriol compared to vehicle and control. The histopathology showed that Calcitriol could significantly lessen kidney damage in contrast to vehicle and control.

Conclusion: This work concluded that Calcitriol significantly reduced RIRI damage in rats due to their pleiotropic effects.

Keywords: *Calcitriol, RIRI, Bcl-2, BAX, mTOR.*

1. 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 & Janabi, 2024).** Numerous clinical scenarios, including organ transplantation, heart and vascular surgery, shock, drug-induced ischemia, and sepsis, can result in IRI **(White & Hassoun, 2011; Kanagasundaram, 2014).**

Since it is directly related to graft rejection, IRI is regarded as one of the main obstacles in organ transplantation. IRI is the cause of 10% of early transplant failures. High rates of acute and chronic graft rejection are also associated with IRI **(De Oliveira et al., 2019).**

The first ischemic insult during IR causes tissue damage and/or death, which is mostly dictated by the degree and length of the blood flow disruption. Reactive oxygen species (ROS) are then produced as a result of reoxygenation, which initiates IRI events and causes a severe inflammatory response, apoptosis, and necrosis of irreparably damaged cells **(Kezić et al., 2017).**

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 & Janabi, 2024b).** By recruiting leukocytes, up-regulating adhesion molecules, and producing mediators such as cytokines, chemokines, ROS, and eicosanoid, inflammatory cells can exacerbate kidney damage **(Hadi et al., 2023).**

TNFα and IL1β are two significant cytokine examples. One of the principal pro-inflammatory mediators or cytokines, TNFα is mostly produced by activated macrophages, but it can also be released by other innate and adaptive immune cells, including mast cells, eosinophils, T and B lymphocytes, neutrophils, and natural killer cells **(Olszewski et al., 2007).** Additionally, cells other than immune cells—such as neurones, adipose tissues, heart myocytes, endothelial cells, fibroblasts, and mesangial cells in glomeruli—form and release it **(Olszewski et al., 2007; Locksley et al., 2001).**

The activation of macrophages by TNFα can result in the release of other cytokines, including IL6, IL-1β, and IL-8 **(Oehadian et al., 2004).**TNFα also plays a significant part in growth regulating, cells and tissues differentiation and apoptosis and also cell cycle **(Locksley et al., 2001; Islam et al., 2017).**

Innate and adaptive immune cells emit IL-1β, a pro-inflammatory mediator believed to be a member of the IL-1 family, during inflammatory reactions. Numerous cell and tissue types produce and secrete the endogenous polypeptide cytokine IL-1β, which plays a crucial role in the aetiology and pathophysiology of AKI and other disorders as pancreatitis **(Ghazi et al., 2019).** IL-1β can be synthesised and released primarily by circulating monocytes, but it can also be produced in low amounts by kidney parenchymal cells under certain conditions, as well as in large quantities by natural killer cells, neutrophils, macrophages, and dendritic cells within tissues **(Netea et al., 2014; Garlanda et al., 2013).** In order to become the active form of IL-1β, which is made up of 153 amino acids, proteolytic activation, an enzyme process, is required to transform the pro-IL-1β, which is inert in nature and has 266 amino acids **(Netea et al., 2014**).

According to the type of body cell, IL-1β is activated under specific intrinsic or extrinsic causes or dangers by a variety of enzymatic activation processes, including the release of reactive oxygen species (ROS) from mitochondria during cell ischaemia reperfusion injury, the leakage of proteases enzyme from lysosomes, and changes in the intracellular concentration of calcium and potassium ions (increased Ca ions influx and K efflux). The production and activation of IL-1β by injured kidney cells initiates the recruitment, activation, and infiltration of more innate and adaptive immune cells by binding to its receptor (IL1R1). Additionally, it causes renal epithelial cells to release more cytokines and chemokines. Numerous research articles have reported these findings **(Leaf et al., 2016; Anders, 2016).** 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 such as lipids and proteins of the cell membrane, carbohydrates, thiols, and DNA. Nonetheless, ROS, particularly H2O2, can benefit tissues mostly through their typical role in cell signalling. As a result, a cell's ROS levels need to be strictly controlled **(Korkmaz & Kolankaya, 2009).**

Instead of employing cyclooxygenases to biosynthesise arachidonic acid, free radicals catalyse the production of advantageous compounds called isoprostanes. Isoprostanes are trustworthy markers of oxidative stress, according to a recent study by the National Institutes of Health (NIH) in the United States **(Gomes et al., 2021).** Due to its high specificity and stability, F2-isoprostane is now thought to be one of the most useful indicators in vivo for evaluating oxidative stress and lipid peroxidation **(Kadiiska et al., 2004).** Therefore, the main and most crucial method to avoid tissue damage during renal ischaemia reperfusion is to use ROS scavengers and antioxidant agents to inhibit or block this pathological pathway or prevent the production of free radicals. This will also shield the tissues from harm and death **(Giovannini et al., 2001).**

The macromolecular protein phosphatidylinositol 3-kinase\protein kinase B\mammalian target of rapamycin (PI3K/Akt/mTOR) signalling pathway is a key mechanism that regulates a wide range of cellular activities. It is also crucial for a number of physiological processes and pathological reactions **(Mengqin et al., 2022).**

The PI3K/Akt/mTOR signalling system is essential for protecting kidney cells from damage by improving cell survival, proliferation, and metabolism. By attaching to their particular receptors and triggering phosphoinositide 3-kinase, or PI3K, several growth factors and cytokines activate this pathway. Then, PI3K-generated phosphatidylinositol (3,4,5)-trisphosphate

(PIP3) attracts Akt (protein kinase B) to the plasma membrane, where it fully activates **(Huang & Tindall, 2007).** Once activated, Akt phosphorylates and deactivates downstream substrates, including the pro-apoptotic proteins caspase-9 and BAD **(Song et al., 2005).** This anti-apoptotic activity is particularly beneficial in the context of acute kidney injury (AKI), as renal tubular cells are particularly susceptible to apoptotic cell death. Studies have demonstrated that PI3K/Akt pathway stimulation can significantly reduce apoptosis in renal tubular cells following ischaemia or nephrotoxic shocks **(Liu et al., 2013).**

Furthermore, autophagy, cell division, and proliferation are all regulated by the mTOR (mammalian target of rapamycin) component of this system. mTOR comes in two complexes: mTORC1 and mTORC2. mTORC1 promotes protein synthesis and inhibits autophagy, whereas mTORC2 regulates cytoskeletal structure and cell survival **(Laplante & Sabatini, 2012).** Deregulation of autophagy has been associated with the progression of renal fibrosis in chronic kidney disease (CKD). Activation of the PI3K/Akt/mTOR pathway can control autophagy and prevent renal fibrosis by maintaining cellular homeostasis and reducing oxidative stress **(Kimura et al., 2011).**

Apoptosis is one of the last processes that takes place in the kidney's ischaemic damaged parenchymal tissues. Both normal undesired cells and diseased cells eventually undergo apoptosis, which is regarded as a programmed cell death **(Hotchkiss et al., 2009).**

Physiologic and pathologic apoptosis are two categories of apoptosis. Normal circumstances, such as the removal of damaged or dangerous cells or those that age or become useless, can trigger physiological apoptosis. However, pathologic apoptosis can eliminate undesirable diseased cells that experience significant DNA degradation, such as those subjected to cytotoxic medicines, radiation, viruses, cancer cells, and severe injury from ischaemia and hypoxia **(Vinay et al., 2018).**

Both of the two main apoptotic routes rely on the activation of caspase enzymes. These pathways are **a)** intrinsic or mitochondrial and **b)** extrinsic or death receptor **(Hotchkiss et al., 2009).**

By interacting with the pro-apoptotic proteins BAX and BAK, the anti-apoptotic protein Bcl-2 blocks many apoptotic triggers and increases cell survival **(Czabotar et al., 2013; Delbridge et al., 2016).** By stabilising and balancing the mitochondrial membrane, regulating its permeability, and preventing the release or leakage of death chemicals such cytochrome c, apoptosis can be controlled or regulated **(Moldoveanu et al., 2014).** Other biological functions of Bcl-2 include controlling the dynamics of mitochondria, regulating the fusing of mitochondrial membranes, and controlling the release of insulin and other metabolic activities in the pancreatic beta cells **(Luciani et al., 2012).**

Bcl-2 level or activity dysregulation, misbalancing, or a defect or damage to the Bcl-2 gene will all result in an imbalance between cell survival, division, and death, which will ultimately cause tumour growth, particularly in tissues that exhibit high division activity, such as breast, lung, and prostate cancer, melanoma, and chronic lymphocytic leukaemia **(Al-Zubaidy et al., 2022b).**
Bcl-2 is regarded as a good biomarker that should be measured during this model of studies (renal I/R model in rats) to assess the severity of injury and to estimate the protection role of the treatment because it plays a crucial role in preventing the apoptosis of parenchymal kidney cells during renal I/R **(Qin et al., 2015a).**

Related to proteins in the Bcl-2 family, BAX is regarded as a pro-apoptotic endogenous agent that triggers apoptosis through the intrinsic pathway by encouraging the caspase cascade to trigger apoptosis and increasing the permeability of the mitochondrial membrane to release cytochrome c **(Westphal et al., 2010).** In many renal illnesses, BAX plays a destructive role by inducing necrosis and apoptosis, which in turn causes cell death and kidney fibrosis. These consequences can be avoided by inhibiting BAX activity using anti-apoptotic drugs **(Docherty et al., 2005; Mao et al., 2008; Konstantinidis et al., 2012).**

BAX is normally present in the cytoplasm, but when apoptosis is triggered, it undergoes a conformational shift and transfers to the organelle membrane, particularly the mitochondrial membrane **(Pierrat et al., 2001; Wolter et al., 1997).** As a result, the mitochondria will produce cytochrome c and a number of pro-apoptotic proteins. Cytochrome c will then trigger the activation of caspase-9, which is a component of the intrinsic cascade of apoptosis. In order to initiate the intrinsic apoptotic process, the active form of caspase-9 will activate caspase-3, which will then intensify the other caspase cascade **(Weng et al., 2005).** BAX is regarded as an essential biomarker to evaluate the degree of damage to kidney parenchymal tissues and to estimate the protection and treatable effects of anti-apoptotic agents used in these conditions because of its critical role in inducing apoptosis during renal I/R injury and exacerbating other kidney diseases **(Westphal et al., 2013).**

Calcium homeostasis and bone metabolism depend on calcitriol, the active form of vitamin D3. Its pharmacodynamics includes its complex interactions with several target tissues, including as the kidneys, bones, and intestines **(Holick, 2007).** Calcitriol enhances intestinal absorption of dietary calcium and phosphate by increasing the synthesis of transporters such as TRPV6 and NaPi-IIb and calcium-binding proteins such as calbindin **(Bouillon et al., 2019; Christakos et al., 2010).** In the kidneys, calcitriol reduces the excretion of calcium and phosphate by increasing their reabsorption in the renal tubules **(Dusso et al., 2005).** Additionally, it has been shown that calcitriol has immunomodulatory qualities. It influences the differentiation

and function of T cells and dendritic cells, which may have implications for inflammatory and autoimmune diseases **(Pérez-López et al., 2012; Adams and Hewison, 2008).**

2. MATERIAL AND METHOD

Site and Ethical Consideration of the Research

The study was done in the department of pharmacology and toxicology \ 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.

Animal Grouping

28 mature Wister Albino rats weighing between 220 and 350 grammes 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 anaesthetic and surgical treatments for ischaemia and reperfusion without ischaemia reperfusion induction. Blood samples and renal tissues were gathered.
2. **Control group:** following a 30-minute bilateral renal ischaemia and a median laparotomy performed under anaesthesia on all seven rats, renal tissues and blood samples were taken two hours following reperfusion **(Yahiya et al., 2023; Bussmann et al., 2014; Zhou et al., 2000).**
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 ischaemia for 30 minutes and reperfusion for two hours **(Alaasam et al., 2024; Ahmed et al., 2021).** At last, both kidneys were removed.
4. **Calcitriol group:** Three days before to the induction of RIRI **(Chen et al., 2018),** all seven-albino rats received an intraperitoneal injection of Calcitriol 2 mcg/kg **(Fu et al., 2013)**. They then experienced bilateral renal ischaemia for 30 minutes and reperfusion for two hours **(Alaasam et al., 2024; Ahmed et al., 2021).** At last, both kidneys were removed.

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 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.

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 **(Tweij et al., 2022).** The left kidney was cut sagittaly into 2 halves. The first half was kept in deep freeze for biomolecular 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 **(Solubility: In DMSO: 50 mg/ml)** as descripted by manufacturer (Medchemexpress).

Assessment of Tissue TNFα, IL1β, F2 isoprostane and KIM1

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 **(Dwivedi et al., 2017; Stokman et al., 2010).** 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, IL1β, F2 isoprostane and KIM1 by ELISA Sunlong kit.

Assessment of Tissue mTOR Gene Expression by RT-qPCR

1. Total RNA Extraction Using Easy-spin™ (DNA free) Total RNA Extraction Kit.
2. cDNA Synthesis (Using AddScript cDNA Synthesis Kit).
3. Preparation of Primers.
4. Primers Used in this Study **(Osqueei et al., 2023; Kunst et al., 2012).**

Host	Gene		5'-3'	Product (bp)	Accession number	Reference
Rattus	mTOR	F	ACGCCTGCCATACTTGAGTC	113	XM_03289 4667.1	**Osqueei et al., 2023**
Rattus		R	TGGATCTCCAGCTCTCCGAA			
Rattus	GAPDH	F	ATGACTCTACCCACGGCAAG	89	NM_01700 8	**Kunst et al., 2012**
Rattus		R	CTGGAAGATGGTGATGGGTT			

5. Protocol of GoTaq® RT-qPCR System for Real-Time qPCR (Gene expression assay).

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% **(Shi et al., 2018).**

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 deparaffinized, 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 counterstain. 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% **(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, was used to perform multiple comparisons across all groups. To determine if there were statistically significant differences between the different study groups in the mean H.score for IHC-P and the total severity score (mean score) for histological renal abnormalities, the Kruskal-Wallis test was utilised. All comparisons and tests were considered statistically significant if $P < 0.001$.

3. RESULTS

Calcitriol Improve Renal Function Parameter

Rats in control and vehicle groups exhibited a significant increase in tissues level of KIM1 in comparison with sham group. Calcitriol pretreatment group was significantly reduced the kidney tissues content of KIM1 comparing with control and vehicle groups (Figure 1).

Calcitriol Attenuated Oxidative Stress and Alleviate F2 isoprostane in Renal Tissue

In our experimental research, we showed that the renal tissue level of F2 isoprostane in sham group was significantly ($p < 0.001$) lower than that level in both control and vehicle groups. The renal tissue level of F2 isoprostane of AD pretreated group was significantly ($p < 0.001$) lower than that level in both control and vehicle groups (Figure 2).

Calcitriol Decreased the Inflammatory Markers in Renal Tissue (TNFα and IL1β)

Protein expression of the inflammatory mediators, TNFα and IL1β, were increased significantly in kidney homogenate of control and vehicle rats in comparison with sham rats. Three consecutive days of IP injection of 2 mcg/kg of AD significantly diminished the expression of TNFα and IL1β in comparison with control and vehicle rats (Figure 3 and 4).

Calcitriol Upregulated Bcl-2 Expression

In this experimental investigation, we demonstrated that the renal tissue Bcl-2 expression of the control group was significantly ($p < 0.001$) greater than that of the sham group. Additionally, compared to the sham group, the renal tissues of the vehicle group showed significantly ($p < 0.001$) higher Bcl-2 expression. One explanation for this increase in Bcl-2 expression in the vehicle and control groups relative to the sham group is that ischaemic tissues used it as a defence or compensating mechanism to stop the apoptotic process. The AD pretreatment group's renal tissue Bcl-2 level was significantly ($p < 0.001$) greater than the control and vehicle groups' levels (Figure 5 and 6).

Calcitriol Downregulated BAX Expression

In the course of our investigation, we discovered that the pro-apoptotic biomarker (BAX) was significantly ($p < 0.001$) less expressed in the renal tissues of the sham group than it was in the vehicle and control groups. The renal tissue level of BAX of AD pretreated group was significantly ($p < 0.001$) lower than those levels in both control and vehicle groups (Figure 7 and 8).

Calcitriol upregulatred the kidney tissues expression of mTOR gene

We conducted in this animal study that there is no considerable variation in renal tissue mTOR gene expression ($p > 0.001$). Furthermore, the renal tissue content of mTOR of AD pretreated group was significantly ($p < 0.001$) higher than those levels in both control and vehicle groups (Figure 9).

Calcitriol 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 ($P < 0.001$). AD pretreated group showed little histological change in contrast to the control and vehicle groups ($P < 0.001$) (Figure 10 and 11).

4. DISCUSSION

IRI is regarded as one of the most significant variables that significantly affects the morbidity and mortality of numerous illnesses, including sepsis, ischaemic stroke, AKI, and MI. Additionally, IRI is seen as a significant problem in some circumstances that can impact healing and clinical outcomes, such as organ transplantation and major surgery. Ischaemia reduces the blood flow to the essential organs, which can lead to hypoxia (low oxygen concentration), a reduction in the supply of nutrients, and the accumulation of CO2 and debris. Micro-blood vessel dysfunction and structural alterations will result from prolonged ischaemia and hypoxia. A large volume of blood will flow to the ischaemic organ after fast reperfusion, which can result in a number of difficulties for that organ. One of the issues that can irritate the tissues and cause more challenges, ROS production, and apoptosis is the inflammation process **(Malek & Nematbakhsh, 2015; Hadi et al., 2017).**

So in our thesis, we estimated the nephroprotective effect of **Calcitriol**, against control renal IRI experimentally.

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

Our animal experiment demonstrated that pretreatment with calcitriol prior to ischaemia induction significantly ($P < 0.001$) reduces the level of KIM1 in renal tissues as compared to the levels in the vehicle and control groups. This finding suggests that calcitriol has a protective effect on renal tissues and function parameters after renal IRI development in a rat model. This result is consistent with previous research. Few research described how calcitriol affected KIM1. According to a recent experimental study, calcitriol pretreatment can lower the urine level of KIM1 in a group of rats that have had renal ischaemia reperfusion **(Huo et al., 2022).**

Calcitriol's Impact on the Kidney Parenchyma

In contrast to the vehicle and control groups, this animal study shows that pretreatment with the active vitamin D3, calcitriol, prior to ischaemia induction significantly ($P < 0.001$) reduces the degree of kidney damage. On the other hand, the vehicle and control groups' mean score intensity indicated severe kidney impairment, but the Calcitriol-pretreated group's mean score intensity projected mild to moderate injury. Our results are consistent with previous studies.

A recent animal study found that administering calcitriol for four months to a group of early weaning baby rats whose mothers had taken high doses of Losartan during the lactating period to induce renal injury could prevent the severity of Losartan-induced nephrotoxicity and preserve the normal nephrogenesis process for those baby rats **(Deluque et al., 2020).**

Effect of Calcitriol on the Inflammatory Mediators (TNFα and IL-1β)

This study demonstrated that calcitriol pretreatment before renal ischaemia induction can significantly ($P < 0.001$) reduce

the levels of inflammatory substances (TNFα and IL-1β) in ischaemic renal tissues, in contrast to the concentrations of inflammatory cytokines in the vehicle and control groups.

According to this study, calcitriol reduces inflammation in kidney tissues that have undergone reperfusion and ischaemia. The results above are consistent with a number of earlier investigations. According to one study on animals, calcitriol consumption can reduce intestinal inflammation brought on by AKI by suppressing the production of genes linked to inflammation, such as TNFα and IL-1β, in the colon **(Huo et al., 2023).**

Effect of Calcitriol on F2 Isoprostane, Oxidative Stress, and Lipid Peroxidation

This laboratory animal study discovered that pretreatment with calcitriol prior to renal ischaemia reperfusion induction can significantly ($P < 0.001$) reduce the level of F2 isoprostane in ischaemic renal tissues, in contrast to the concentrations of this oxidative stress biomarker in the vehicle and control groups. This study suggests that calcitriol has an anti-oxidative effect and reduces lipid peroxidation and ROS production in damaged renal tissues that have undergone ischaemia and reperfusion.

The data from this animal work coincides with many works. An animal study was conducted on nursing Holstein cows that had mastitis as a result of an interventional Streptococcus uberis bacterial infection. It was shown that giving those cows calcitriol could reduce the amount of F2 isoprostane in their milk as well as the oxidative stress condition brought on by bacterial infection. Accordingly, calcitriol can lessen the severity of mastitis in such cows and plays an antioxidant effect **(Wells et al., 2023).**

Impact of Calcitriol on Anti-apoptotic Marker (Bcl-2) and Pro-apoptotic Marker (BAX)

This study shown that, in comparison to the vehicle and control groups, pretreatment with calcitriol before the onset of ischaemia reperfusion injury in the kidney can significantly ($P < 0.001$) alter the ratio of BAX/Bcl-2 in these damaged renal tissues. In renal tissues, it can increase the expression of Bcl-2 and decrease the degree of BAX. This suggests that calcitriol can stop necrosis and apoptosis in injured kidney tissues because it possesses antiapoptotic qualities.

These findings align with those of other earlier research. Giving rats with spinal cord injuries calcitriol has been shown to preserve the viability of the spinal cord cells, raise Bcl-2 expression and lower BAX expression, and prevent necrosis and apoptosis, which kill neurone cells **(Zhou et al., 2016).**

Impact of Calcitriol on mTOR Protien

In this animal study, we elucidated that the pretreatment group with calcitriol had considerably ($P < 0.001$) higher kidney tissue concentrations of PI3K\Akt and mTOR gene expression than the vehicle and control groups. According to the data from this animal study, calcitriol protects damaged kidney tissues by upregulating the expression of the protective molecular signalling pathway (PI3K\Akt\mTOR).

Our findings are consistent with those of other studies. According to some recent research on HeLa cell lines, calcitriol may increase cytotoxicity in a dose-and time-dependent manner, which could result in HeLa cell growth arrest. This was accomplished by activating the PI3K\Akt\mTOR molecular pathway, which inhibited autophagy and changed the homeostasis of mitochondrial biogenesis **(Setiawan et al., 2022).**

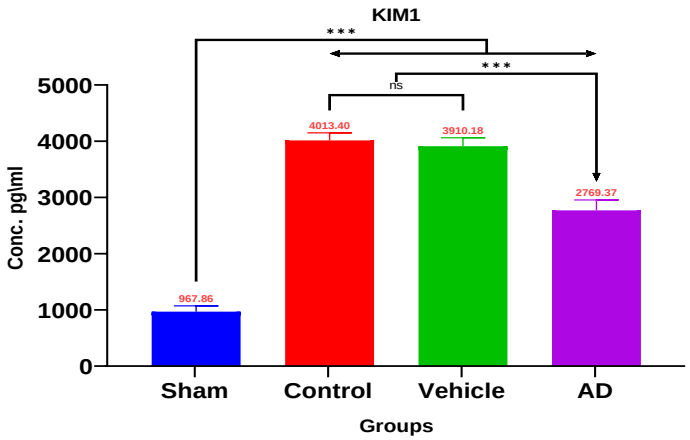

Figure (1): The statistical analysis of KIM1 concentrations mean (pg/ml) in renal tissues in the four experimental study groups at the finishing of the research (No of rats = 7 in each study group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

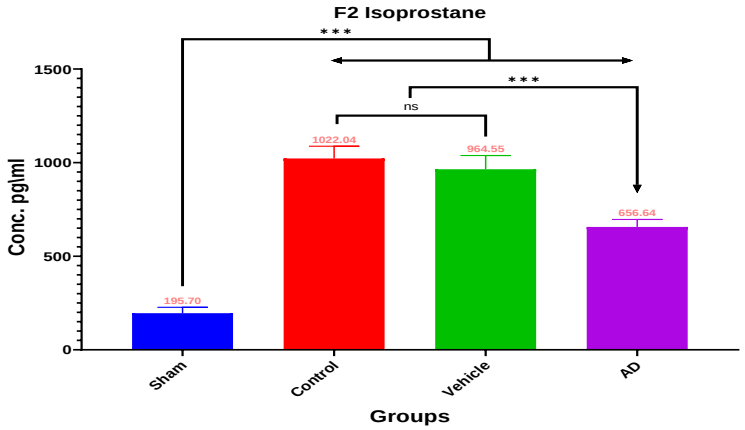

Figure (2): The statistical analysis of F2 Isoprostane concentrations mean (pg/ml) in renal tissues in the four experimental study groups at the finishing of the research (No of rats = 7 in each study group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

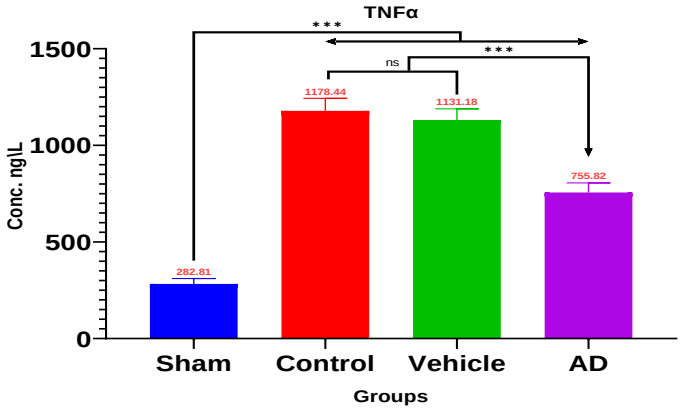

Figure (3): The statistical analysis of TNFα concentrations mean (ng/L) in renal tissues in the four animal study groups at the finishing of the research (No of rats = 7 in each study group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

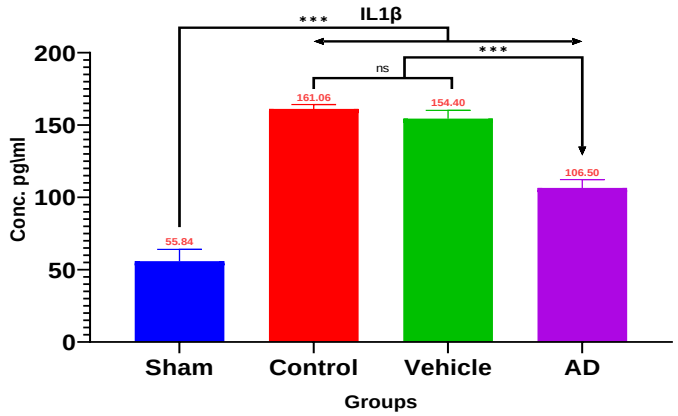

Figure (4): The statistical analysis of IL-1β concentrations mean (pg/ml) in renal tissues in the four animal study groups at the finishing of the research (No of rats = 7 in each study group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

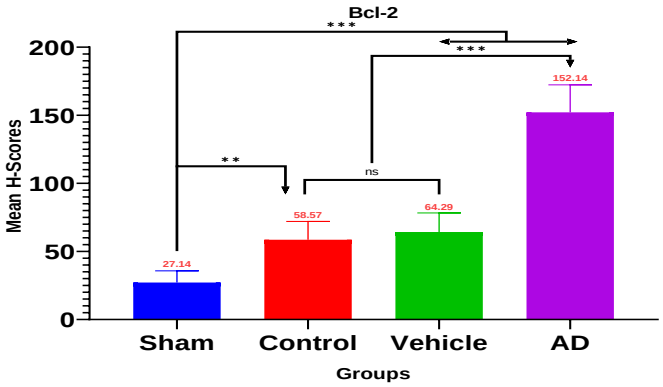

Figure (5): Mean H.scores of Bcl-2 in renal tissue of the four experimental groups at the end of the study (No of animals = 7 in each group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

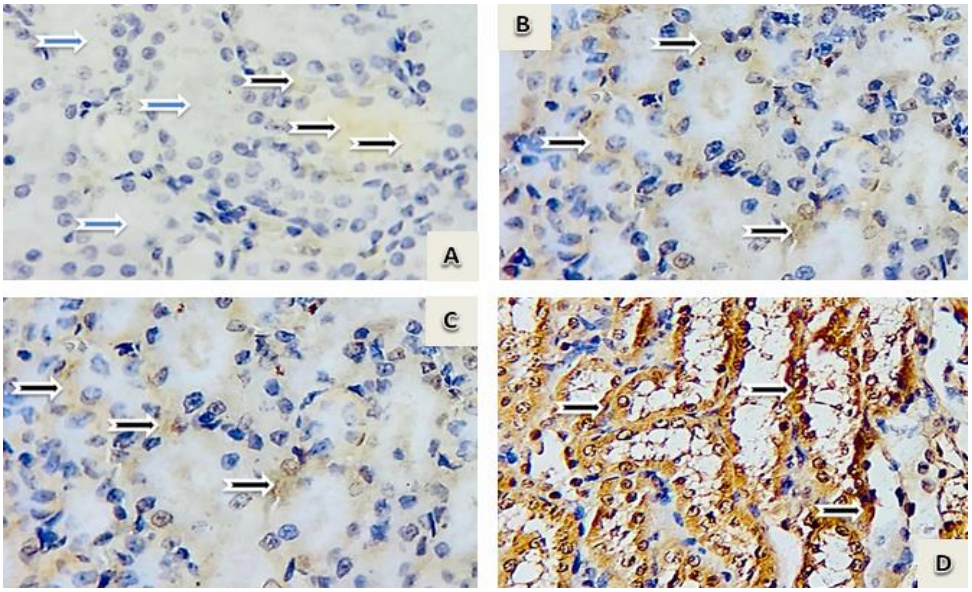

Figure (6): A) A cross section of left kidney represented a negative to slightly positive cytoplasmic brown stain of Bcl-2 protien (blue and black arrows respectively) × 400. Sham group. B) A cross section of left kidney showed a slightly positive cytoplasmic brown stain of Bcl-2 protien (black arrows) × 400. Control group. C) A cross section of left kidney appeared a slightly positive cytoplasmic brown stain of Bcl-2 protien (black arrows) × 400. Vehicle group. D) A cross section of left kidney appeared a strong positive cytoplasmic brown stain of Bcl-2 protien (black arrows) × 400. AD treated group.

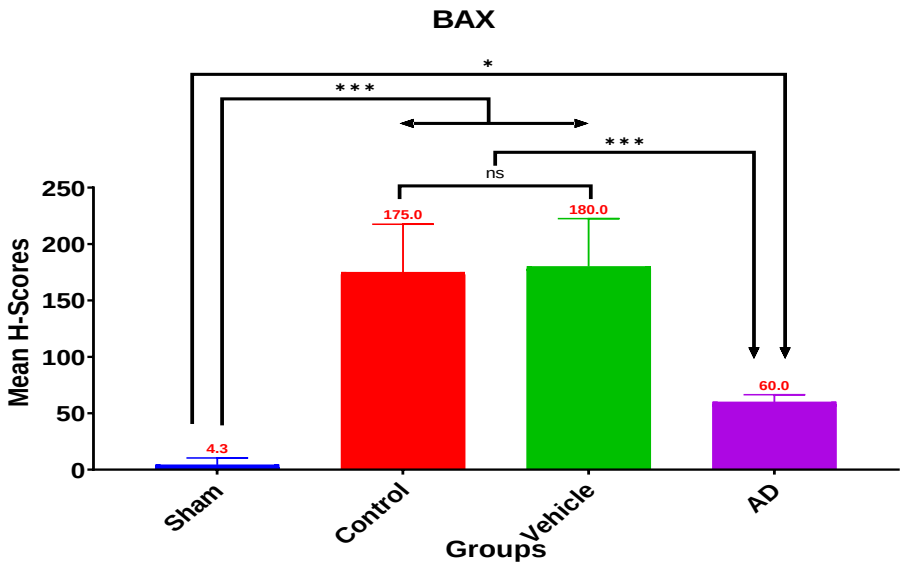

Figure (7): Mean H.scores of BAX in renal tissue of the four experimental groups at the end of the study (No of animals = 7 in each group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

AD vs. Sham group, *P.value = 0.027

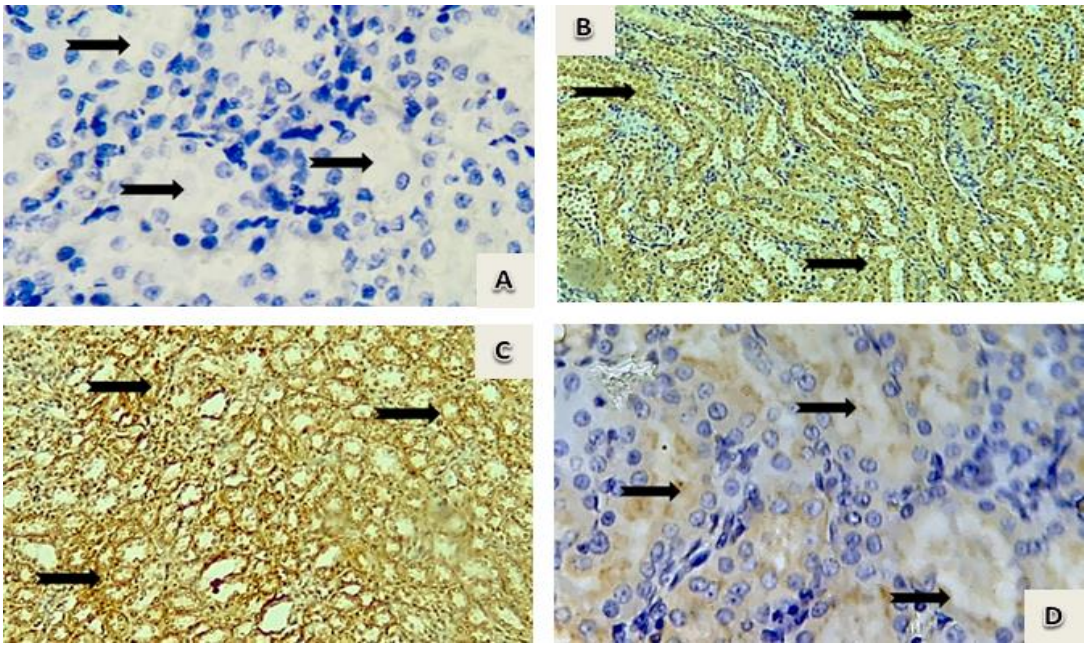

Figure (8): A) A cross section of left kidney showed BAX negative cytoplasmic stain (black arrows) × 400. Sham group (Zero H.Score). B) A cross section of left kidney showed BAX highly intense positive cytoplasmic stain, brown stain (black arrows) × 100. Control group. C) A cross section of left kidney showed BAX highly strong positive cytoplasmic stain, brown stain (black arrows) × 100. Vehicle group. D) A cross section of left kidney showed BAX slightly brown stain (black arrows) × 400. AD treated group.

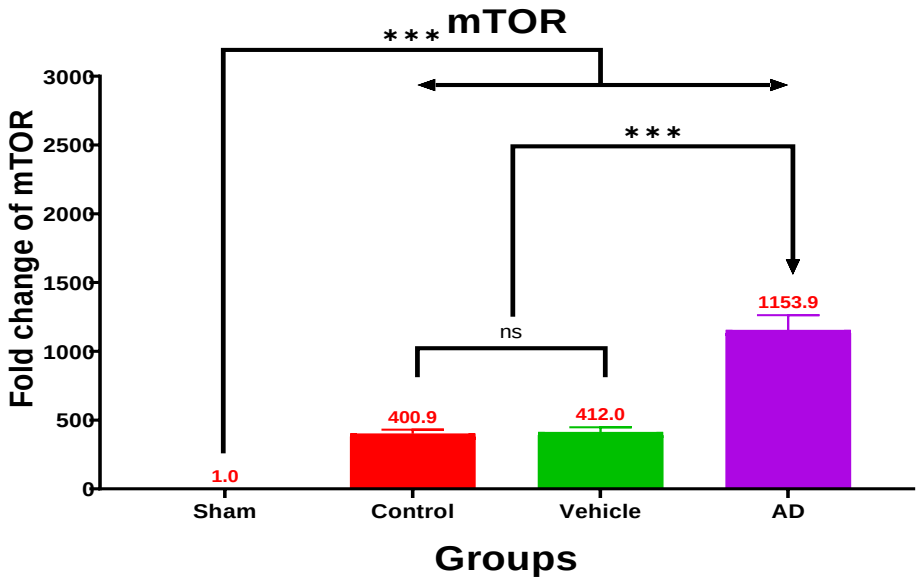

Figure (9): Mean of fold change of mTOR gene in renal tissue of the four experimental groups at the end of the study (No of animals = 7 in each group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

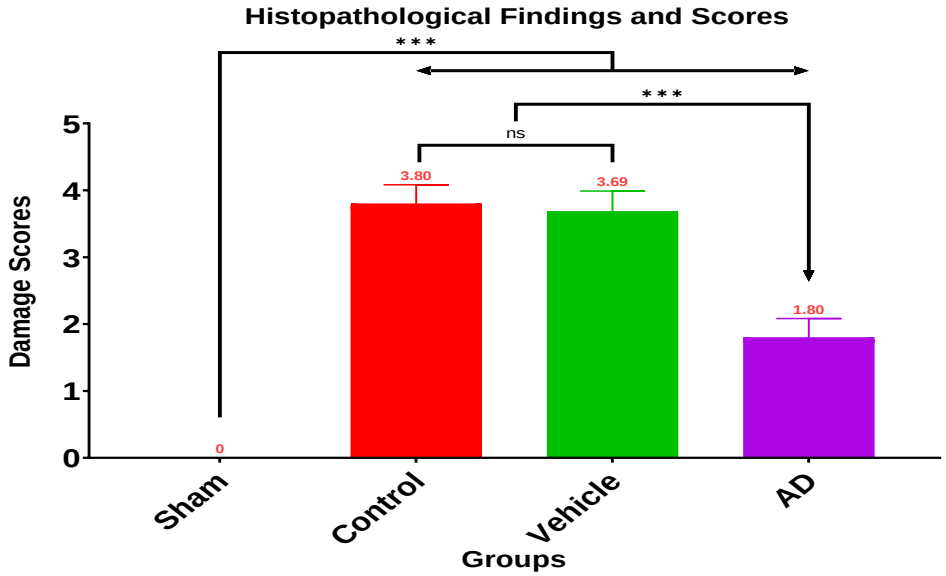

Figure (10): Score severity mean of renal tissue histopathology of the four experimental groups at the end of the study (No of animals = 7 in each group).

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

AD vs. vehicle & control groups, ***P.value < 0.001

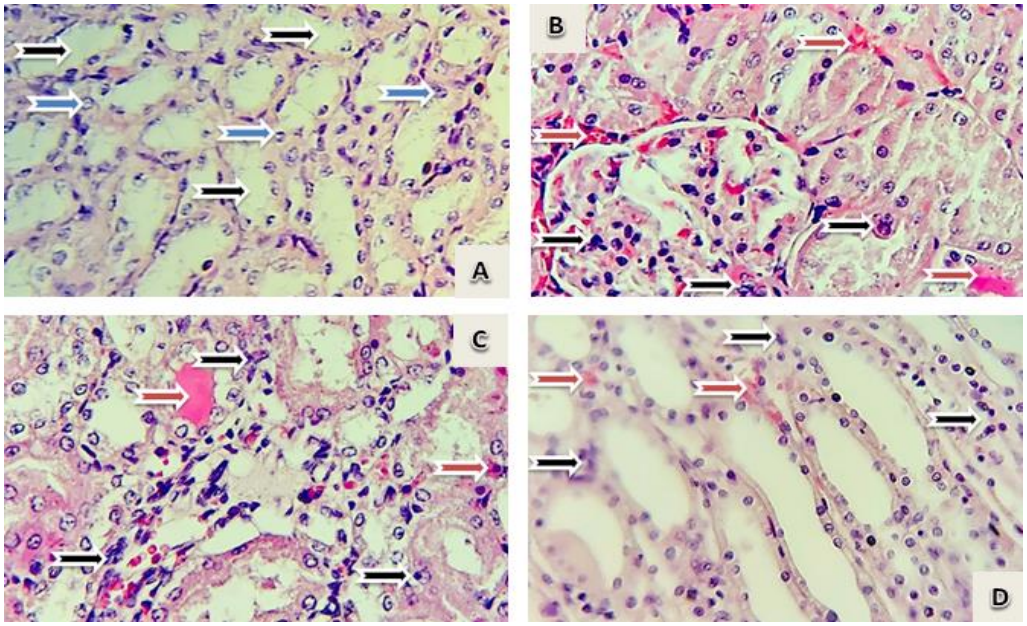

Figure (11): A) A microscopic cross section of left kidney represented normal tissues histology, normal renal tubules (black arrow), normal cell size (blue arrow) and there are no cast formation, cells odema or loss of brush boarder. Sham group. H & E stain × 400. B) A microscopic cross section of left kidney represented score 4 tissues modifecations including severe cellular odema, cytoplasmic eosinophilia (black arrows) and strongly eosinophilic cast (red arrows). Control group. H & E stain × 400. C) A microscopic cross section of left kidney represented score 4 tissues modifecations including severe cellular odema, cytoplasmic eosinophilia (black arrows) and strongly eosinophilic cast (red arrows). Vehicle group. H & E stain × 400. D) Microscopic cross section of left kidney represented score 2 tissues modefications including few eosinophilic cast (red arrows), moderate cellular odema and tubular dilatation (black arrows). AD treatment group. H & E stain × 400.

5. 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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