

# Ranolazine Protects the Kidney from Ischemia/Reperfusion Injury in Adult Male Rats by Modulation of Inflammatory and Oxidative Pathways and Suppression of Notch2/Hes1 signaling pathway

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## Abstract

**Background:** Renal ischemia and reperfusion is the main cause of acute injury of the kidney and associated with increase morbidity, mortality, and long stage hospitalization. Renal ischemia and reperfusion occur in approximately 10-20% of kidney transplant recipients. If ischemia and reperfusion occur, this will induce oxidative stress, inflammatory process and apoptosis.

**Objectives:** This research aimed to investigate the possible nephroprotective effect of ranolazine in renal ischemia reperfusion injury in rat model by targeting Notch2/Hes1 signaling pathway.

**Materials & Methods:** 24 adult male Wistar Albino rats were divided randomly into 4 groups (six rats in each group): Sham group underwent laparotomy without induction of ischemia reperfusion, Control group (Rats underwent 30 minutes bilateral renal ischemia followed by 2 hours reperfusion, Vehicle group (same as in control group + DMSO), Ranolazine group (same as in control group + 30mg/kg ranolazine). After 2 hrs of reperfusion, the kidney and blood were harvested. Blood sample was used to assess serum creatinine and blood urea nitrogen (S.cr & BUN). Renal tissue was used to assess IL-1 $\beta$ , HMG box1, F2-isoprostane and Notch2/Hes1 as well as histological examination.

**Results:** At the end of experiment, S.cr, BUN and renal tissue level of HMG box1, IL-1 $\beta$ , F2-isoprostane, and Notch2/Hes1 were significantly increased in control group as compared with sham group. Histopathology study demonstrated severe kidney injury in the control group as compared with sham group. Kidneys of rats in ranolazine treated group demonstrated functional and histological improvement by significant decrease S.cr., BUN, renal tissue level of HMG box1, IL-1 $\beta$ , F2-isoprostane, Notch2/Hes1, and severity score of tubular injury also significantly decreased in ranolazine treated group as compared with control and vehicle groups.

**Conclusion:** From the overall results, ranolazine significantly decreases renal ischemia reperfusion injury in rats which was achieved by modulation of inflammatory response through significant reduction in renal tissue level of HMG box1, IL-1 $\beta$ , and down regulation of Notch2/Hes1 signaling pathway, and anti-oxidant effect via significant reduction in F2- isoprostane level.

**Keywords:** Ranolazine, Ischemia reperfusion injury, Notch2/Hes1, HMG box1, IL-1 $\beta$ , F2-isoprostane.

## Abbreviations

AKI (acute kidney injury), S.cr. (serum creatinine), BUN (blood urea nitrogen), ROS (reactive oxygen species), IL-6 (interleukin-6), IFN- $\gamma$  Interferon gamma), IL-8/CXL1 (Interleukin-8/ Lymphotoxin (CXL1), MCP-1 (monocyte chemoattractant protein-1), RBP-Jk (Recombination signal binding protein for immunoglobulin kappa J region), DMSO (Dimethyl Sulfoxide), IRI (ischemia reperfusion injury), RIRI (Renal ischemia reperfusion injury), PPAR gamma (peroxisome proliferator-activated receptor gamma).

## INTRODUCTION

Ischemia reperfusion injury is a sudden temporary reduction of blood and oxygen supply (hypoxia or anoxia) to the organ followed by restoration of blood supply and re-oxygenation. Deficiency in Oxygen and nutrients in ischemic process will lead to many events include oxidative stress and inflammation [1]. Renal ischemia

reperfusion injury is the main cause of AKI which is associated with increase morbidity, mortality, and long stage hospitalization [2]. Sudden decrease in renal blood flow is happened in many clinical cases: kidney transplantation, renal artery angioplasty, partial nephrectomy, cardiopulmonary bypass, aortic bypass operation, iatrogenic or accidental trauma,

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hydronephrosis, sepsis, and elective urological surgeries [3]. Renal ischemia reperfusion injury stimulates many inflammatory cascades that encourage more renal damage. The major arbiters of inflammation are chemokines which regulate pro-inflammatory cytokines, expression of adhesive molecules infiltration and activation of leukocytes [4]. Restoration of oxygen level during reperfusion will induce formation of reactive oxygen species, cytokines, Chemokines, recruitment activation, adhesion and immigration of neutrophils that will lead to trigger ischemic injury [5]. Neutrophils are the early cellular mediators of local microvascular changes and parenchymal destruction. When Neutrophils reach to the ischemic area, they will release ROS, protease, elastase, myeloperoxidase enzymes, cytokines and other inflammatory mediators [6]. Mitochondria is the main energy source of the human body and is much in proximal tubules of kidney making the renal cortex a central area of oxygen utilization to produce energy [7] Mitochondria may be disturbed in many pathological conditions such as ischemia reperfusion injury by energy stress and increase production of toxic reactive oxygen species which will lead to oxidative stress, intracellular calcium overload, apoptotic and necrotic cell death [8]. Tissue hypoxia that occurs during ischemia will shift metabolic pathway from aerobic to anaerobic which will lead to decrease ATP production and cause lactate accumulation resulting in an intracellular acidosis. ATP level reduction will lead to electrolytes disturbance such as sodium and water influx, intracellular calcium accumulation also hypoxia decreases the antioxidants enzymes activity such as superoxide dismutase, catalase and glutathione peroxidase, and prevent expression of cytochrome c oxidase [9]. One method to assess oxidative damage is to measure lipid peroxidation. F2-isoprostane is widely used to measure lipid peroxidation and it is the most reliable biomarker of oxidative stress in animals and human studies [10]. Isoprostanes are prostaglandin like compounds, in vivo they result from free radicals catalyzed arachidonic acid peroxidation independent of the cyclooxygenase enzyme [11]. Many properties of F2-isoprostane make it a suitable biomarker to assess the oxidative stress in vivo. They are specific products of lipid peroxidation formed on phospholipids and then by the action of phospholipase A2 they will be released. Isoprostane can be quantified in all biological tissues and fluids [12]. Ischemic acute kidney injury stimulates series of pro-inflammatory pathways and through production of soluble mediators and activation of the innate and adaptive immune system of the host; it will facilitate other organs injury in the heart, lungs, brain, liver, and gut including multiple inflammatory pathways by increasing soluble pro inflammatory mediators expression [113]. High mobility group box 1 (HMGB 1) is a substantial endogenous injury related molecule, highly distributed in mammalian lymphoid tissues, liver, brain, lung, heart, spleen, kidney and other organ tissues. It is found in nucleus of most organs, but mainly found in cytoplasm in liver and brain tissues [14]. HMG box1 protein is one of Toll-like receptors endogenous ligand and represents a key factor of inflammatory response caused by ischemia reperfusion injury [15]. One study states that HMG box1 was released into the cytoplasm of neurons within 1 hour of brain ischemia. Other study reported that HMG box1 was trans located into the

cytoplasm of the ischemic core of striatum as early as thirty minutes after injury. Binding of HMG box 1 to its receptors will activate downstream pathway that excites inflammation by up regulation IL- 1B, IL-6 and TNF-alpha and other cytokines [16]. Interleukin 1 B (IL- 1B) is a key member of interleukin 1 family that mediates inflammatory response via interleukin 1 receptors leading to fever, Neutrophils migration, T-cell differentiation and function, recruitment of natural killer cells and macrophages response [17]. Many studies demonstrated the functional role of IL-1 $\beta$  in different models of IRI such as heart, liver, kidney, gut, brain, limb and lung ischemia and reperfusion [18]. Interleukin 1 activates many types of cells to produce abundant inflammatory cytokines and chemokine including IL-6, TNF alpha, IFN- $\gamma$ , IL-8/CXCL1, MCP-1, and IL-1 itself. This IL-1 dependent inflammatory cascade leads to inflammatory cell infiltration and subsequent tissue damage play a central role in pathogenesis and progression of renal ischemia reperfusion injury [19]. Parts of the kidney which are most affected by ischemic injury are proximal tubules S3 segment and Medullary thick ascending limb of loop of Henle because of these parts found physiologically in low oxygen area [20]. Renal ischemia leads to loss of cytoskeletal completeness. There is brush borders shedding in the proximal tubules, loss of cell polarity and adhesion molecules position, dysfunction of sodium-potassium ATPase transporter pump and loss B integrin proteins of plasma membrane. Cytokines lead to damage of cell matrix and cohesion dependent on B integrin. Cell-cell communication will be deactivated by disruption of adhesive tight junctions [21]. Acute tubular necrosis (ATN) is the most common cause of AKI. ATN is mostly occurs due to acute ischemia, nephrotoxins, or sepsis. ATN leads to reduction of glomerular filtration rate and suddenly increase serum creatinine and blood urea nitrogen, and injury to epithelial cells of renal tubules [22]. Notch 2/ Hes 1 pathway in renal ischemia: Notch is a protein and cell surface Trans membrane receptor [23]. Four types of notch receptor in mammals include (notch-1, notch-2, notch-3, and notch-4) which are activated when ligand from adjacent cell binds to the extracellular part of notch receptor. Five ligands available for notch receptors include (Delta like ligand Dll-1, Dll-3, Dll-4), jagged-1 and jagged-2 [24]. When tissues exposed to hypoxia and toxic damage, this will activate the expression of ligand from adjacent cells to bind to the extracellular domain of notch receptor and activate it leading to proteolytic cleavage of intracellular domain and enter to the cell nucleus [25]. When notch intracellular domain which referred as NICD entered to the nucleus, it will bind with recombinant signal binding protein-Jk and activate it to start gene expression [26]. NICD-RBP-Jk will activate expression of downstream pathway including hairy enhancer of split 1 (hes-1) and lead to NF- $\kappa$ B gene transcription. In adult, Notch pathway in kidney is highly reduced but will be reactivated in acute and chronic renal injury promoting inflammation and apoptosis [27]. In mammals, the Hes family is composed of seven genes (Hes 1-7), only Hes 1, Hes 5 and 7 are controlled by the Notch signaling pathway [28]. Notch signaling is up regulated and increase in acute and chronic renal disease. It was firstly observed by Kobayashi et al that the expression of Delta-1, Notch-2

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/Hes1 is up-regulated in rat ischemia and reperfusion injury [29]. Ranolazine is derived from piperazine. In United States and Europe is approved as a second line agent in the treatment of chronic stable angina. It reaches to peak plasma concentration at about 2 – 6 hours after administration [30]. Peak plasma concentration of ranolazine from oral solution or immediate release capsule is obtained within 1 hour after administration [31]. During ischemia, inside sodium channel stays open leading to accumulation of sodium in the cell and activate sodium/calcium exchange causing calcium overload in the cell this pathway results in mismatch between oxygen demand and supply. So ranolazine by inhibit inward sodium flow contributes to the balance of oxygen demand and supply [32]. Modification of energy metabolism is the main therapeutic purpose to improve the efficacy of O<sub>2</sub> utilization during ischemia. Metabolic effect which acts as the main mechanism of anti-ischemic effect of ranolazine is by partially inhibit fatty acid oxidation and the energy metabolism will be shifted from fatty acid oxidation toward glucose oxidation. Glucose oxidation needs less oxygen than fatty acid oxidation so ranolazine will improve the ischemia by inhibit ischemic reperfusion injury also other mechanism of ranolazine may be acts by decrease reactive oxygen species formation [33].

## **MATERIALS AND METHODS**

The study was done in the department of pharmacology and therapeutics and Middle Euphrates Unit for Cancer Researches, Faculty of Medicine, and 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.

### **Design of study**

Adult male Wistar Albino rats had been used with 16 – 24 weeks of age and 250 – 350 g in weight were purchased from center of control and pharmaceutical research – Ministry of health / Baghdad and transported from Baghdad to Najaf by an equipped car with cage and air-condition and has been harbored in the animal house at the college of Science – University of Kufa with a temperature controlled at 24±2 c° and humidity range from 60 – 65% with the fit 12 hour light : 12 hour dark cycle for two weeks before start of the procedure. In this study, the rats were divided randomly in to four groups (6 rats in each group) and as the following:

- 1- Control group: Rats were anesthetized with intraperitoneally 100mg/kg ketamine and 10mg/kg xylazine [34-35]. After anesthesia they were undergone midline laparotomy incision to expose the right and left renal pedicles and subjected to bilateral renal ischemia for 30 minute followed by 2 hours reperfusion [36-37].
- 2- Sham group: Rats underwent the same anesthetic and midline laparotomy procedure without induction of ischemia reperfusion.
- 3- Vehicle group: Rats were injected intraperitoneal DMSO 30 minutes before ischemia as a vehicle for ranolazine according to solubility instructions of manufactured company (Medchemexpress/USA), then anaesthetized and underwent the same surgical and ischemic procedures as in control group.
- 4- Ranolazine treated group: Rats were pretreated with intraperitoneally 30mg/kg of ranolazine [38-39]. Ranolazine was injected at 30 minute before ischemia

[40-41], and then the rats were anaesthetized and exposed to 30 minute of bilateral renal ischemia and 2 hours reperfusion.

### **Experimental procedure**

After anesthesia with ketamine and xylazine hydrochloride as mentioned above, rats were placed on its back and fixed their limbs and tail with a medical plaster to assure their stability during surgery, after that hair in the abdomen had been shaved and the area disinfected. When the rats are adequately anesthetized, the abdomen will be exposed by making a midline laparotomy incision in order to expose the right and left renal pedicles. Bilateral renal ischemia is made by non traumatic micro vascular clamps then covers the abdomen by warm and moist gauze. After 30 minutes, the blood had been returned to the kidneys by removing the clamps and can be assured visually then close the abdominal cavity incision with a suture type 3/0 silk. 1 cc of isotonic sodium chloride 0.9% solution pre-warmed at 37c ° had been used on the abdomen to avoid dehydration. After 2 hours of reperfusion, the animals were euthanized by taking the blood from the heart by cardiac puncture and harvesting both kidneys for parameters examination [42]. It was demonstrated by Haynes R et al., 2014 that the ischemia of kidney for more than 20 minutes will lead to damage. If the ischemia continues for 20 to 40 minutes, it can be reversible; while reperfusion after ischemia for more than 40 minutes can result in permanent damage [43].

### **Preparation of ranolazine**

The drug was prepared immediately before using by reconstituted it with dimethyl sulfoxide (DMSO) according to instructions of manufactured company (Med Chem Express, USA).

### **Collection of blood sample for measurement of renal function**

At the end of the procedure, rats were still anesthetized; about 2.5-3 milliliter of blood was taken directly from the heart. The blood sample was put in a plane tube at 37c° without anticoagulant, then it will centrifuged at 3000rpm for 10 minutes to obtain serum which is used for determination of urea and creatinine [44].

### **Tissue preparation for IL-1B, HMG box 1, and F2-Isoprostane measurement**

The kidney was washed with cold isotonic sodium chloride solution 0.9% to remove any blood, stored in deep freeze at -80 Celsius. Then renal section was taken and homogenized with a high intensity ultrasonic liquid processor in 1:10 W/V phosphate buffered saline which contains 1% Triton X-100 and 1% of protease inhibitor cocktail. For good homogenization, further breakdown of the cell membranes was achieved by subjection of the homogenate to high intensity ultrasonic liquid processor [45]. The homogenates were centrifuged at 3000 rpm for 20 minutes at 4 C and the supernatant were used for determination of IL-1B, HMG box1, and F2-isoprostane according to the manufactured company of Eliza kits (Bioassay Technology Laboratory).

### **Tissue preparation for Histopathology**

Renal tissues of left kidney acquired after sacrifice of the rats had been washed with cold isotonic sodium chloride

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solution 0.9% to remove red blood cells or clots then fixed in 10% formalin and processed in paraffin tissue blocks and microscopic section was taken to involve the renal cortex and pelvis. After that, section of 5 micrometer thickness had been taken from the blocks and stained with hematoxylin-eosin dye. Histopathological changes were estimated for cellular swelling, cytoplasmic eosinophilia, tubular dilation, loss of brush borders, development of protein casts, epithelial cell de squamation in to the lumen, inflammatory reaction, cell lysis and necrosis [46]. The histopathological test was made in 100 to 400X of original magnification. The severity score of tissue damage was examined by a Histopathologist in a blinded method and scored by the percentage of tubular damage as the following scores [47]:

- 0: no damage.
- 1: less than 25%.
- 2: 25 – 50%.
- 3: 50 – 75%.
- 4: more than 75%.

### Immunohistochemistry technique

Immunohistochemistry was performed to assess Notch2 and Hes1 in kidney tissue. 5µm paraffin embedded sections were stained by utilizing immune staining procedure. Briefly, sections were subject to deparaffinized, rehydration, antigen repairing by exposed to retrieval buffer, and inhibiting endogenous peroxidase activity by 3% H<sub>2</sub>O<sub>2</sub>. The sections were incubated with Notch2 or Hes1 polyclonal antibody (Diluted 1:200 for Notch2, Elabscience) and (1:100 for Hes1, Bioassay) overnight at 4 °C. 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 Notch2 or Hes1 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% [48].

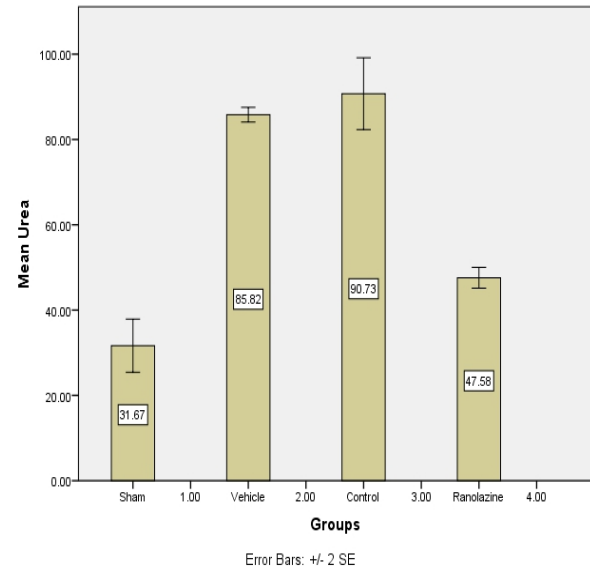
### Statistical Analysis

Statistical analyses were done by SPSS version 26. The data were expressed as Mean ± SEM. ANOVA (analysis of variance) was used for comparisons of multiple groups followed by post-hoc test using Bonferroni correction. For the Immunohistochemical results and histopathological changes of renal tissue, Kruskal-Wallis test was used to determine statistical significance of the difference among the multiple groups as mean score for histopathological changes of renal tissue and mean H score for immunohistochemical results. Statistically in all tests, P value ≤ 0.001 was considered significant.

## RESULTS

### Ranolazine ameliorate renal function

Rats in control and vehicle groups exhibited a significant increase in serum urea and creatinine level comparing with sham group. Ranolazine pretreatment was significantly reduced the two markers of kidney function

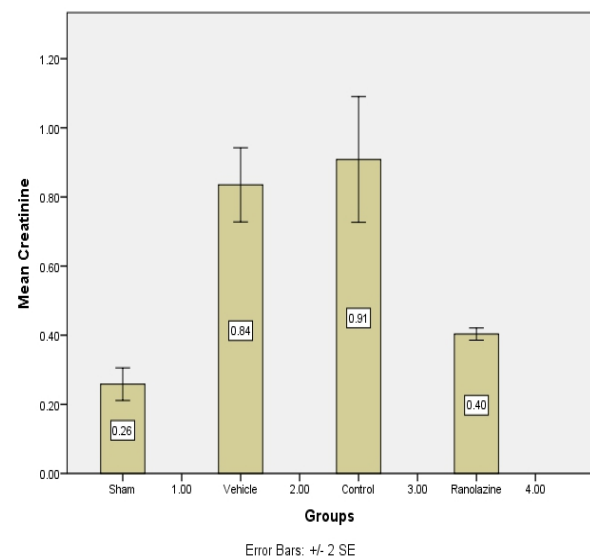


comparing with control and vehicle groups (Figure 1,2).

**Figure 1.** Mean serum level of urea (mg/dl) of the four experimental groups at the end of the experiment (number of rats in each group is 6)

-Sham vs. vehicle and control groups, P value =0.00001 (Significant)

-Ranolazine vs vehicle and control groups, P value =0.00001 (Significant)



**Figure 2.** Mean serum level of creatinine (mg/dl) of the four experimental groups at the end of the experiment (number of rats in each group is 6)

-Sham vs. vehicle and control groups, P value =0.00001 (Significant).

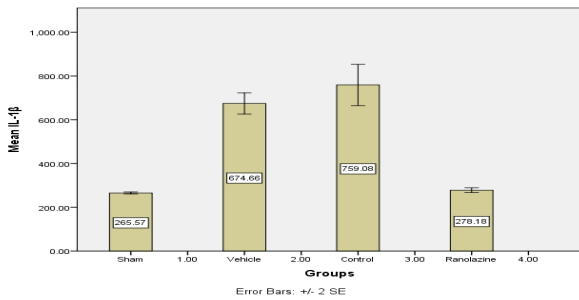
-Ranolazine vs vehicle and control groups, P value =0.00001 (Significant).

### Ranolazine attenuated oxidative stress (F2-isoprostane) in renal tissue

Rats in control and vehicle groups exhibited a significant increase in renal tissue level of F2-isoprostane comparing with sham group. Ranolazine pretreatment was significantly decreased the renal tissue level of F2-isoprostane comparing with control and vehicle groups

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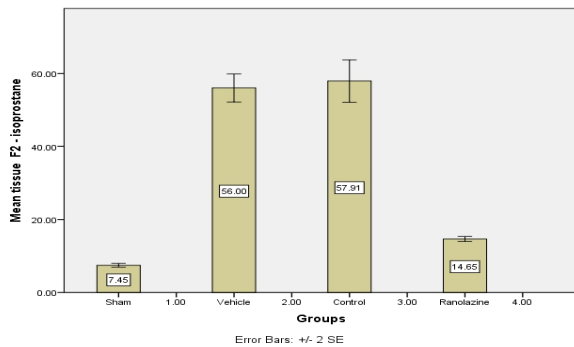
(Figure 3).



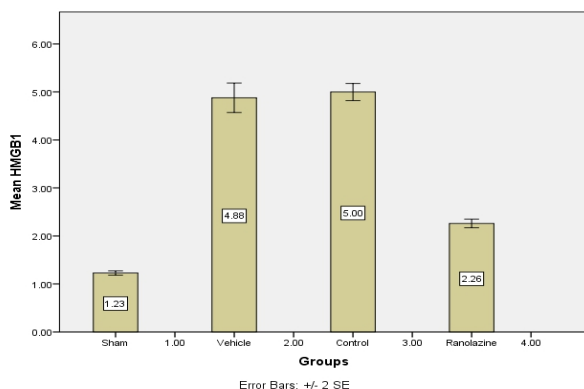
**Figure 3.** Mean tissue level of F2 isoprostane (ng/L) of the four experimental groups at the end of the experiment (number of rats in each group =6)  
 -Sham vs vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value =0.00001 (Significant).

**Ranolazine decreased inflammatory markers in renal tissue**

Renal tissue level of HMG box1 and IL-1 $\beta$  was significantly increased in control and vehicle groups as compared with sham group. 30mg/kg Ranolazine pretreated group demonstrated significantly decrease the protein expression of inflammatory mediators HMG box1 and IL-1 $\beta$  as compared with control and vehicle groups (figure 4 and 5).



**Figure 4.** Mean tissue level of HMGB-1 (ng/ml) of the four experimental groups at the end of the experiment (number of rats in each group =6)  
 -Sham vs vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value =0.00001 (Significant).

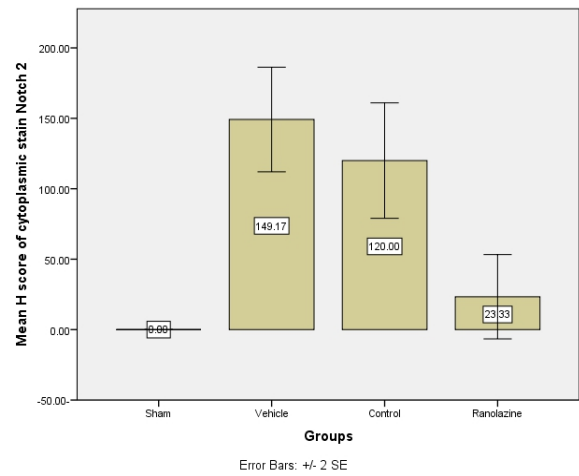


**Figure 5.** Mean tissue level of IL-1 beta (pg/ml) of the

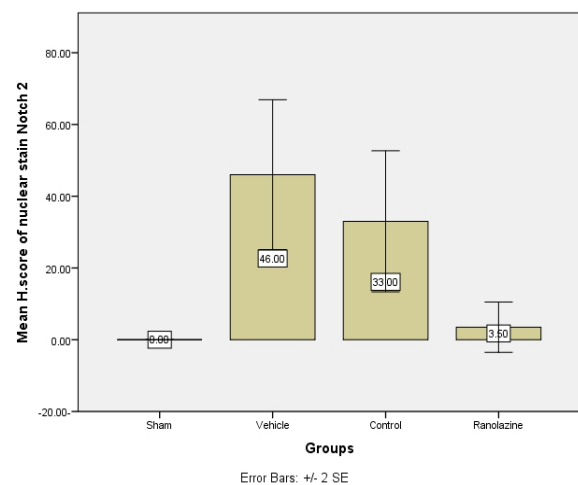
four experimental groups at the end of the experiment (number of rats in each group =6)  
 -Sham vs. vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value=0.00001 (Significant).

**Ranolazine down regulated the Notch2/Hes1 expression**

The expression of Notch2/Hes1 (Figures 6, 7, 8, 9, 10) was measured by immunohistochemical technique. The results showed that the level of protein expression of Notch2/Hes1 was much greater in control and vehicle groups (p value  $\leq$  0.001) as compared with sham group. On the other hand, treatment with ranolazine significantly reduced the protein expression obviously.

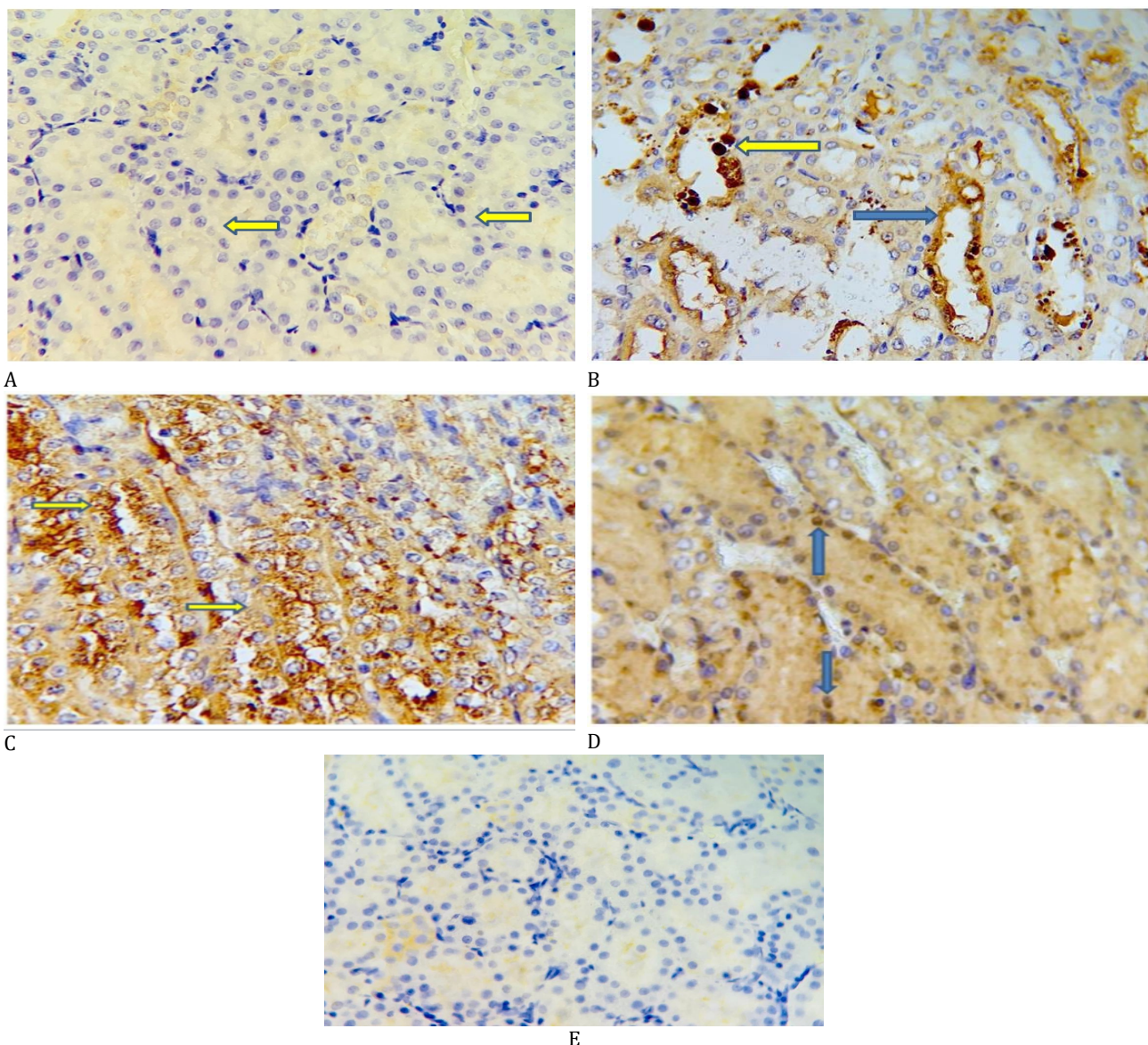


**Figure 6.** Mean H score of cytoplasmic staining of Notch2 renal tissue level of the four experimental groups at the end of the experiment (number of rats in each group =6)  
 -Sham vs. vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value =0.00001 (Significant).



**Figure 7.** Mean H score of nuclear staining of Notch2 renal tissue level of the four experimental groups at the end of the experiment (number of rats in each group =6)  
 -Sham vs vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value =0.00001 (Significant).

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**Figure 8.** Immunohistochemical staining for Notch2

A: Notch2 negative stain in sham group, yellow arrows demonstrate normal renal tubules, mean H score of cytoplasmic and nuclear staining = 0 (picture was magnified X400).

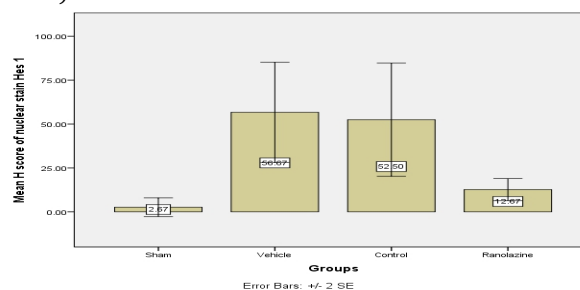
B: Notch2 positive nuclear stain (yellow arrow) mean H score = 33 and cytoplasmic stain (blue arrow) mean H score = 120 in control group (X400).

C: Notch2 strong positive cytoplasmic stain (yellow arrows) in vehicle group due to inflammation and apoptosis, mean H score of cytoplasmic stain with Notch2 = 149.17 (picture was magnified X400).

D: Notch2 positive nuclear stain (blue arrows) in vehicle group, mean H score of nuclear stain with Notch2 = 46 (picture was magnified X400).

E: Notch2 negative stain in ranolazine group, demonstrates significant reduction of inflammation and apoptosis by ranolazine group as compared with control and vehicle groups, mean H score of cytoplasmic stain in ranolazine group = 23.33, and mean H score of nuclear stain in ranolazine group = 33 (picture was magnified

X400).

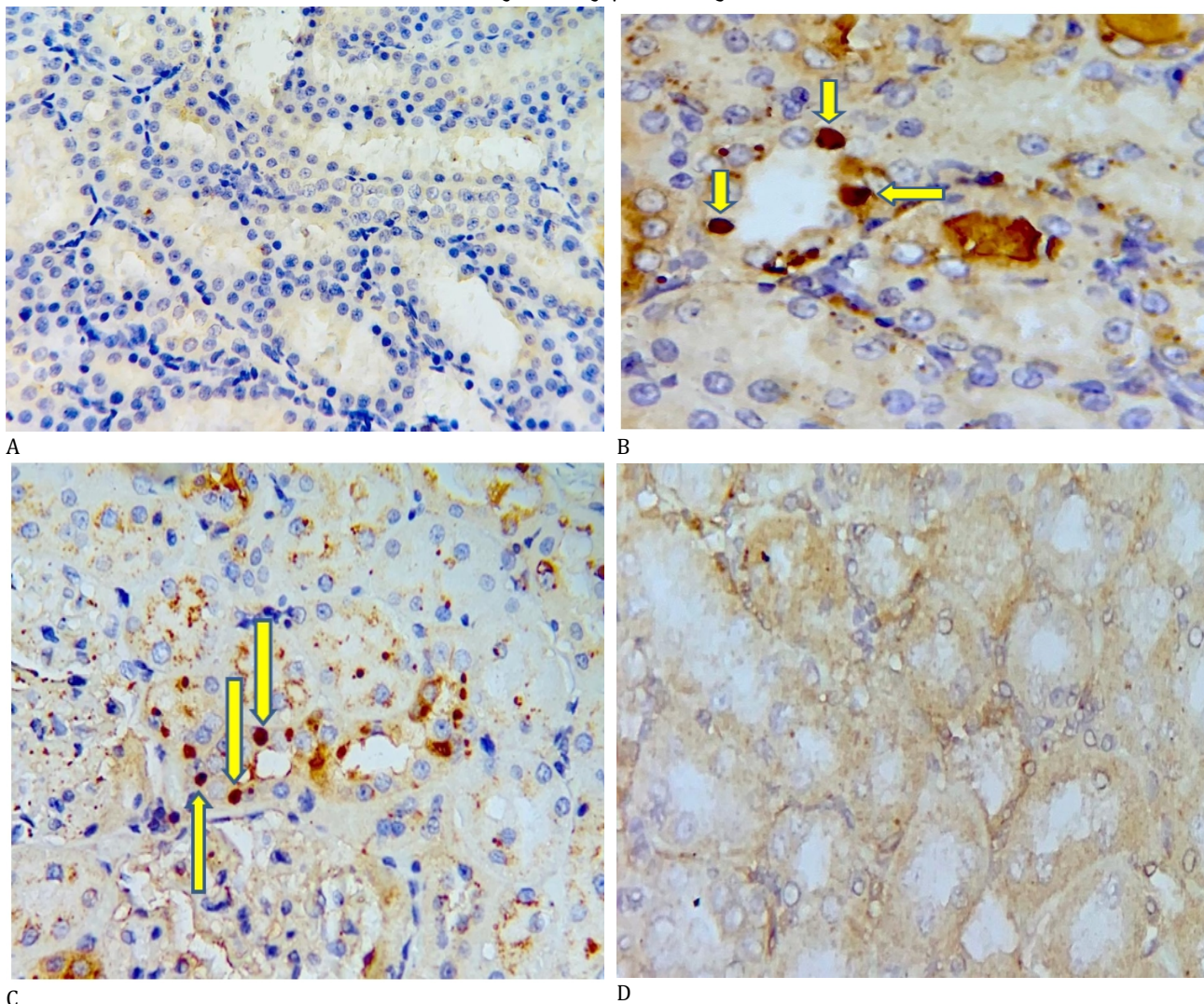


**Figure 9.** Mean H score of nuclear staining of Hes1 renal tissue level of the four experimental groups at the end of the experiment (number of rats in each group = 6)

-Sham vs vehicle and control groups, P value = 0.001 (Significant).

-Ranolazine vs vehicle and control groups, P value = 0.001 (Significant).

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**Figure 10.** Immunohistochemical staining for Hes1

A: Hes1 negative stain in sham group demonstrates normal renal tubules, mean H score of nuclear stain with Hes1 in sham group= 2.67 (picture was magnified X400)  
 B: Hes1 strong positive nuclear stain (yellow arrows) in control group, mean H score of Hes1 nuclear stain in control group= 52.50 (picture was magnified X400).  
 C: Hes1 positive nuclear stain (yellow arrows) in vehicle group, mean H score of Hes1 nuclear stain in vehicle group= 56.67 (picture was magnified X400).  
 D: Hes1 negative stain in ranolazine group, mean H score of Hes1 nuclear stain in ranolazine group= 12.67, this score demonstrates significant reduction of inflammation by ranolazine group as compared with control and vehicle groups.(picture was magnified X400).

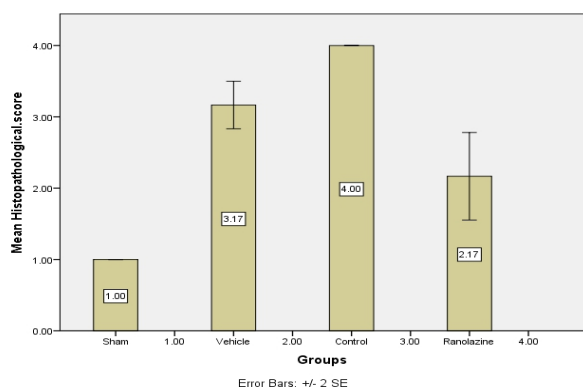
**Ranolazine minimized kidney injury**

Histopathological examination showed mild tubular

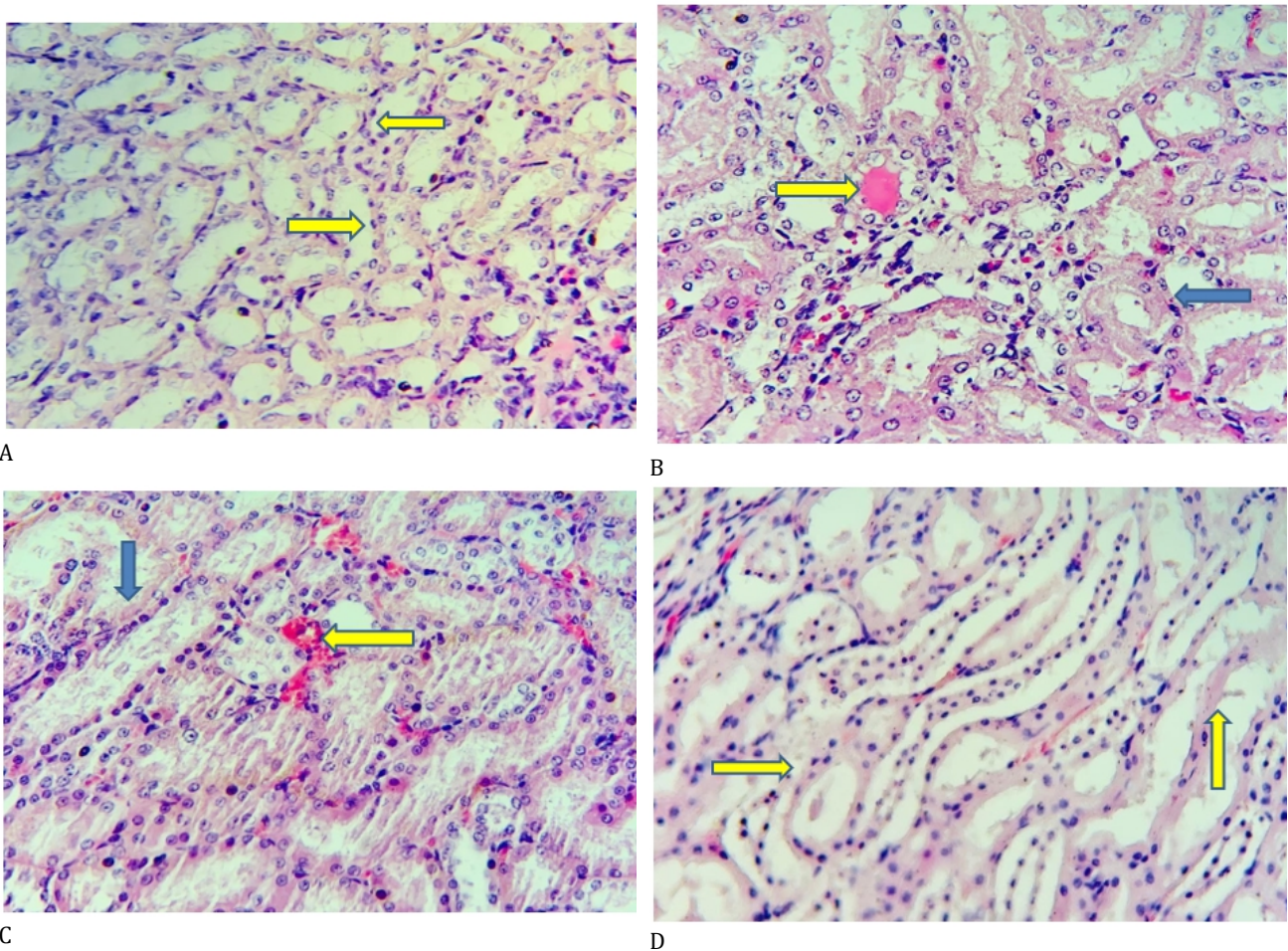
injury in the kidney of sham group. In control and vehicle groups, an increased number of damaged tubules and cell dilatation were noticed in comparison with the sham group (p value ≤ 0.001). Ranolazine pretreated group showed little histological change in contrast to the control and vehicle groups (p value ≤ 0.001) (figure 11&12).

**Figure 11.** Mean Histopathological severity score of renal tissue of the four experimental groups at the end of the experiment (number of rats in each group =6)

-Sham vs. vehicle and control groups, P value =0.00001 (Significant).  
 -Ranolazine vs vehicle and control groups, P value=0.00001 (Significant).



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**Figure 12.** Histopathological examination of renal section

A: photomicrograph of the renal section for sham group demonstrates mild tubular injury (yellow arrows show renal tubules), Mean severity score of tubular injury in sham group = 1. The section stained with haematoxylin and eosin (x400).

B: photomicrograph of the renal section for the vehicle group demonstrates ischemic changes (blue arrow demonstrates cellular swelling and increase cytoplasmic eosinophilia, and yellow arrow demonstrates eosinophilic casts inside lumen), Mean severity score of tubular injury in vehicle group = 3.17. The section stained with haematoxylin and eosin (x400).

C: photomicrograph of the renal section for the control group demonstrates ischemic changes (blue arrow demonstrates cellular swelling and cytoplasmic eosinophilic), yellow arrow shows red blood cells, Mean severity score of tubular injury in control group = 4. The section stained with haematoxylin and eosin (x400).

D: photomicrograph of the renal section for the ranolazine treated group demonstrates mild residual ischemic changes (yellow arrows). Mean severity score of tubular injury in ranolazine group = 2.17, the section stained with haematoxylin and eosin (X 400).

**DISCUSSION**

Renal ischemia reperfusion injury is the main cause of AKI. The main pathological characteristic of AKI is an interstitial inflammation [49]. The damage and dysfunction of mitochondria play a causative factor in pathogenesis of acute kidney injury, especially for injury

and death of tubular epithelial cells. Mitochondrial damage does not only disturb energy metabolism of the cell but also lead to more ROS production and release of pro-apoptotic factors which may result in death of renal tubules [50]. During reperfusion, ROS will be generated lead to oxidative stress and development of RIRI which will lead to ischemic acute kidney failure [51]. Acute kidney injury had been as risk factor to develop CKD with high morbidity and mortality rate which may be reach to Two millions/year worldwide[53]. Renal ischemia is common problem occurs in kidney transplantation, nephron sparing surgery, cardiopulmonary bypass, and hydronephrosis which will result in dysfunction and injury of kidney. So it is important to urgent medical intervention for RIRI to protect against AKI and its results [61].

**Effect of R/I on renal function parameters urea and creatinine**

In this study, Blood urea nitrogen and S. creatinine levels were significantly elevated in control and control vehicle group as compared with sham group. This result is compatible with the study by (Maryam et al., 2019) that showed significantly elevated of s. creatinine and BUN in control and control vehicle groups as compared with sham group in rats exposed to 30 min. bilateral renal ischemia/2 hrs. reperfusion [54].

**Effect of Ranolazine on renal function parameters (Urea and Creatinine)**



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In this study, pretreatment with ranolazine was significantly decrease serum creatinine and BUN as compared with control (I/R) and control vehicle groups. This result demonstrated that ranolazine maintained renal function after ischemia reperfusion injury, to the best of our knowledge; there is no previous study about the effect of ranolazine on serum creatinine and BUN in RIRI.

### **Effect of renal I/R on oxidative stress marker F2 isoprostane**

In this study, the level of F2-isoprostane in renal tissue was remarkably elevated in control and control vehicle groups as compared with sham group.

This result is compatible with the study by **Maryam et al., 2020** who showed that significantly ( $p$  value  $\leq 0.001$ ) elevated level of renal tissue F2-isoprostane in control group as compared with sham group in adult male rats exposed to 30min. bilateral renal artery occlusion followed by 2 hrs reperfusion [55].

### **Effect of Ranolazine on oxidative stress marker F2 isoprostane**

In this study, ranolazine pretreated group showed significantly ( $p$  value  $\leq 0.001$ ) lower level of renal tissue F2-isoprostane as compared to control and control vehicle group. Schwemer T.F. et al., 2019 showed that the urine level of oxidative stress marker F2-isoprostane is significantly decreased after 6 weeks of treatment with ranolazine in patients with myocardial infarction as compared with control group in randomized RIMINI trial [56]. El Amrani FB et al., 2014 Showed that ranolazine inhibits oxidative damage by increase anti-oxidant Copper/Zinc-superoxide dismutase in astrocytes primary culture in neuronal inflammation, autoimmune and neurodegenerative diseases [57]. McCormack, 1996 found that ranolazine has a beneficial effect in myocardial ischemia and reperfusion in rats by stimulate glucose oxidation and decrease fatty acid oxidation, which will result in improvement of ATP production and decrease the synthesis of hydrogen ions, lactate, and harmful intermediates of fatty acyl [58].

### **Effect of renal I/R on inflammatory mediator HMG-box1**

In this study, the renal tissue level of high mobility group box 1(HMGB1) was significantly elevated in I/R (control group) and control vehicle group as compared with sham group. Yasmeen et al., 2019 ; Aseel et al., 2019 demonstrated that HMG box1 level is significantly elevated in mice and rats exposed to 30min. bilateral renal artery occlusion followed by 2 hrs reperfusion [27], [37]. HMG box1 released during ischemia of kidney and liver and acute lung injury activates cell signaling pathway through toll like receptors 2, 4, and 9 and lead to up regulation of pro inflammatory cytokines and chemokine which will cause tissue damage [59].

### **Effect of Ranolazine on inflammatory mediator HMG box1 level**

In this study, the renal tissue level of HMG box1 was remarkably lowered in ranolazine treated group as compared with I/R (control) and control vehicle group, this effect may contribute to the anti inflammatory effect of ranolazine. To the best of our knowledge, there is no previous study about the effect of ranolazine on HMG box1 in RIRI.

### **Effect of renal I/R on inflammatory mediator IL-1 $\beta$**

In this study, the level of IL-1B in renal tissue was remarkably increased in control (I/R group) and control vehicle group as compared with sham group. This result is in agreement with the study which demonstrated that the level of IL-1B was significantly elevated in control (I/R group) and control vehicle group as compared with sham group in rats exposed to bilateral occlusion of renal artery for 30min. followed by reperfusion for 24hrs [60]. Also it was found that the level of IL-1 $\beta$  in renal tissue was significantly elevated in control (I/R group) and control vehicle group as compared with sham group in rats exposed to bilateral renal ischemia for 30 min. followed by 2 hrs reperfusion [61]. IL-1 $\beta$  plays an important role in the induction of inflammation by stimulate production of pro-inflammatory molecules such as interleukin 6 and prostaglandin E2 also it leads to expression of leukocytes adhesion molecules and matrix degrading metalloproteinase resulting in the inflammation and remodeling of tissues [62].

### **Effect of Ranolazine on inflammatory mediator IL-1 $\beta$**

In this study, it was found that IL-1B level in renal tissue is significantly decreased in Ranolazine treated group as compared with I/R (control group) and control vehicle group. This effect suggests that ranolazine may improve renal ischemia reperfusion injury through its anti-inflammatory effect on IL-1B. This significant effect of ranolazine on IL-1B level may be related to anti inflammatory effect of ranolazine and is compatible with the studies that showed ranolazine has cardioprotective and neuroprotective effect by inhibiting inflammatory process. Ranolazine ameliorates endothelial function by decrease inflammatory mediators IL-1 and TNF-alpha and increase anti-inflammatory PPAR gamma. Ranolazine showed a predominant anti-inflammatory effect by decreasing inflammation in atherosclerosis and post ischemic complications like re infarction ,infarct expansion and also inhibit cardiovascular events such as restenosis after percutaneous coronary intervention [57], [63-64]. To the best of our knowledge, there is no previous study about ranolazine effect on inflammatory mediator IL-1B in RIRI.

### **Effect of renal I/R on Notch2/Hes1**

Notch signaling is activated and up regulated in kidney injury and glomerular disease [65]. Notch2 staining in immunohistochemistry was detected and up regulated in the cytoplasm and nucleus of the control group during renal ischemia and reperfusion; while high percent view of the samples that are stained with Hes1 antibody were found in the nucleus of the proximal tubules in the outer medulla and cortex after ischemia and reperfusion injury in the kidney [66]. In this study, Notch2/Hes1 was significantly ( $p$  value $<0.05$ ) elevated and up regulated in control and control vehicle groups as compared with sham group. This result is consistent with the result by **Yasmeen et al., 2019** who showed that significantly over expression of Notch2/Hes1 protein in control and control vehicle groups as compared with sham group in mice exposed to 30min./2hrs. bilateral renal artery occlusion [27].

**Renfa Huang et al., 2011** found the inhibition of Notch2/Hes1 signaling pathway by DAPT treatment in rats exposed to renal ischemia/reperfusion injury resulted in decrease the severity of damage in renal

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tubules, improve renal function and reduce the concentration of pro inflammatory mediators (TNF-alpha and IL-6) so decrease inflammation and apoptosis [67].

## Effect of Ranolazine on Notch2/Hes1 level

The present study showed that significant decrease in protein expression of Notch2/Hes1 in ranolazine treated group as compared with control and control vehicle groups. To the best of our knowledge, there may be no previous study about the effect of ranolazine on Notch2/Hes1 pathway in RIRI.

## Effect of renal I/R on renal parenchyma

In this study, the total severity score of tubular injury in control (I/R) and control vehicle groups was significantly ( $p$  value  $\leq 0.001$ ) higher than the score of sham group. Histological scanning after RIRI demonstrated cellular swelling, cytoplasmic eosinophilia, tubular dilation, loss of brush borders, development of protein casts, and epithelial cell desquamation into lumen, inflammatory reaction, cell lysis and necrosis. This result is compatible with studies by (Aseel Noaman et al., 2019; Shahin Mozaffari et al., 2020) who showed significant increase of total severity score of tubular injury in control(I/R) group as compared with sham group in rats exposed to respectively 30min. bilateral renal ischemia/2hrs reperfusion and 45 min bilateral renal ischemia/24hr. reperfusion [37], [68].

## Effect of ranolazine on renal parenchyma

In the present study, the total severity score of tubular injury in ranolazine treated group was mild kidney injury which is significantly ( $p$  value  $\leq 0.001$ ) lower than the score of control (I/R) and control vehicle groups which were severe kidney injury. This protective effect of ranolazine is due to its anti-inflammatory and antioxidant effect of it which results in decrease the severity of renal injury if it is given before ischemia. Naveena et al., 2018 demonstrated that the histopathologic examination of granulation tissues showed decrease the severity of cellular infiltration in ranolazine treated group as compared to control (I/R) group. This effect is due to decrease the level of inflammatory mediators such as TNF-alpha, C reactive protein, and interleukin 6 and so lead to decrease infiltration of the cells at the site of inflammation [63].

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