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NEPHROPROTECTIVE EFFECTS OF QUERCETIN IN RENAL ISCHEMIA REPERFUSION INJURY IN MICE

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ABSTRACT

The purpose of the present paper is to study the Nephroprotective I effect of Quercttin in renal ischemia reperfusion mice model. The methods are forty male Swiss Albino mice 7-15 weeks weighting 30-35 gram (g) were divided into four groups, ten mice in each group, animal groups are arranged as, group I (Sham group) have been subjected to the same anesthetic and surgical laparotomy but without induction of IRI, Group II (Control group): Exposed to bilateral renal IR procedure under anesthesia, Group III (Vehicle group): Received vehicles sterile dimethyl sulfoxide (DMSO 0 .2 %) alone intraperitoneal 30 minutes before exposed to renal IR procedures under anesthesia, Group IV (Quercttin treated group): Receiving Quercttin in a dose of 50 mg/kg intraperitoneal injection 30 minutes before exposed to IR procedure under anesthesia and served as treated group. Blood samples were collected directly from the carotid vein for determination of serum levels of urea & creatinine, NGAL and Notch level. Bilateral nephrectomy was done and homogenized for measurements of tissue markers (Interleukin6 (IL-6), Toll like receptors 2 (TLR-2), F2 Isoprostane, Bc12 associated X protein (Bax) and B cell lumphoma2 (Bcl2). In this in addition to mean serum levels of (Urea and Creatinine) and mean of plasma NGAL beside the mean of Notch1 Jagged 1 and scores for histopathological changes were significantly (*P<0.05) elevated in control group as compared with that of sham group while in Quercttin treated group the above mentioned parameters were significantly (*P<0.05) decreased, in addition to histopathological changes score and according to the mean tissue level of Bcl-2 the result was controversial. In conclusion, Quercttin possess a renoprotective effects via modulation of the inflammatory, oxidative and apoptotic pathway.

Keywords: Renal Ischemia, reperfusion injury, Quercttin.

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INTRODUCTION

Ischemia is a restriction in blood supply to tissues, causing a shortage of oxygen that is needed for cellular metabolism (to keep tissue alive) which is caused by problems with blood vessels, with resultant damage to or dysfunction of tissue [1]. During Ischemia reperfusion injury (IRI), the damaged tissue produce excessive amount of Reactive oxygen species (ROS) cause oxidative stress which changes mitochondrial oxidative phosphorylation, ATP (adenine triphosphate) depletion, increase intracellular calcium and activation of membrane phospholipids proteases[2]. Oxidative stress is the key mediator of Ischemia Reperfusion (I/R) injury due to the inability of the innate antioxidant defense system to buffer the large burst of free radicals, which ultimately results in membrane lipid per oxidation [3]. Inflammation and apoptosis play an important role in the pathogenesis of IR, inflammation starts during ischemia and accelerates upon reperfusion with endothelial activation, leukocytes recruitment, up regulation of chemokine and cytokines, and activation of the complement system result in cellular dysfunction [4]. Toll-like receptors (TLRs) are a conserved family of cell membrane receptors constitutively expressed within the kidney that are part of the innate immunity system playing an important role as a first response to tissue injury [5]. Although many mechanisms are involved in ischemic acute kidney injury (AKI), apoptosis is one of the most essential mechanisms causing cell death [6]. Neutrophils gelatinase-associated lipocalinis (NGAL) is a small secreted polypeptide increases massively in the kidney and in the first urine output in early ischemic AKI in rats and mice [7].Notch is an integral membrane protein that interacts with membrane-bound ligand either delta-like (Delta it's a ligand for Notch 1-4) (Dll 1/3/4) or serrate-like (jagged (Jag) 1/2) family [8]. Notch-1Jaggged-1 could be biomarkers of kidney damage since it expressed in a wide range of renal diseases like Albuminuria and glomerulosclerosis and Tubulointerstitial fibrosis [9]. Quercttin is a strong antioxidant and anti-inflammatory effects, prominently found in colored fruits and vegetables [10]. Aim of the study: This study was undertaken to determine the possible Nephroprotective effect of Quercttin in renal IRI via interfering with the inflammatory and oxidative pathways.

Materials and Methods

A total of 40 adult male Swiss Albino mice developed 7-15 weeks, with the optimum age being 8 weeks weighing (30-35) gram obtained from Animal Resource Center, the National Center for Drug Control and Researches in

Baghdad. the study protocol was approved by the Animal Ethics Committee (AEC) of Kufa University (no.101) in 2019, Animals were housed in the animal house in university of Kufa with a controlled temperature of 25±10C with alternating 12h light:12 hour (h) dark cycle , animals had free access to standard chow and tap water. Animal care and administration met those required by an applicable international laws and regulations.

Study design

After two weeks of acclimatization forty mice were divided into four groups, each of ten mice. Allocation of animals in their groups was completely random, animal groups were treated as follows:

- Group I (Sham group): Subjected to the same anesthetic and surgical laparotomy but without induction of IR.
- Group II (Control group): Exposed to bilateral renal IR procedure under anesthesia
- Group III (Vehicle group): Received vehicles sterile dimethyl sulfoxide (DMSO 0 .2 %) alone intraperitoneal 30 minutes before exposed to renal IR procedures under anesthesia.
- Group IV (Quercttin treated group): Received Quercttin in a dose of 50 mg/kg intraperitoneal injection 30 minutes before exposed to IR procedure under anesthesia and served as treated group.

Surgical procedure

Optimized pre-operative preparation of the animal for the induction of IRI was performed before animals anaesthetized with ketamine (80 milligram mg/ kilogram kg) and xylazine (4mg/kg) intraperitoneally [11]. Following induction of anesthesia, the abdomen is depilated and disinfected, drops of normal saline is applied to mice eyes to make sure the cornea is protected from drying and trauma , the animal is placed with its back in a position with its head and neck extended to ensure that its airway remains unobstructed [12] . The abdomen is opened with a midline incision by using a wound spreader, the intestines are carefully pushed aside and the kidneys are exposed [13]. The renal pedicle is clamped with micro aneurysm clamps, after only 30 minute time of occlusion the clamps were removed to start reperfusion, a Vicryl 4–0 suture is used to first close the muscle layer, followed by closing of the skin. Sham animals are subjected to the exact same surgical procedure, aside from clamp placement [14]. At the end of reperfusion period the animal is re- anesthetized by giving inject able anesthetic over dose according to the Institute for Animal Studies Veterinarians intraperitoneal to be sacrificed [15]. Prepare to sample collection a needle of the syringe is introduced into carotid vein to aspirate around 2ml of blood for later blood analysis , the kidneys are cut, a portion of the renal tissues were homogenized to be used for tissue measurement while the other portion of the kidney is fixed in 10 % formalin for histological analysis[16].

Samples preparation

Blood sampling

About 2 ml of blood was collected from the carotid vein of each mouse. The first half was placed immediately in a two tubes containing anticoagulant used for determination of Notch-1 Jagged-1 level by flow cytometry and NGAL, while the remaining blood was allowed to clot in an ordinary tube at 37 0C then it was centrifuged at 3000 rpm for 15 minutes then the supernatant (serum) was used for the determination of blood urea nitrogen and serum creatinine levels.

Tissue Sampling for ELISA (Enzyme linked immunosorbent assay)

A portion of kidney tissue weighed and homogenized with a high intensity ultrasonic liquid processor in 1:10 (w/v) phosphate buffered saline that contained 1% Triton X-100 and a protease inhibitor cocktail [17]. The homogenate was centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was collected for determination of IL-6 , TLR2, F2 Isoprostane, Bax and Bcl2 by ELISA with a commercially available ELISA kit.

Tissue Sampling for Histopathology Scoring

A portion of renal tissue was immediately fixed in 10% buffered formalin solution. Examine renal structure by histopathology on paraffin embedded kidney tissue sections stained with Hematoxylin and Eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the kidney. The following light microscopic features were used to assess the histopathological damage. The score (0) represents normal, score (1) represents <25% of the damage of the tubules, score (2) represents 25%_50% of damage of the tubules and score (3) represents >51% of damage of the tubules [18] .

Statistical Analysis

SPSS version 22 was used for statistical analysis, results were analyzed using one-way ANOVA, post-hoc multiple comparisons test (TUKEY) to investigate the difference among groups. The results variability was expressed as mean ± standard error of the mean (SEM). P value of 0.05 was considered statistically significant.

Results

Effect of Renal I/R on Renal Function Test

The results showed a significantly increased in the level of renal blood urea nitrogen (BUN) and serum creatinine (SCRE) in control group as compared with sham group (*p < 0.05). There was insignificant difference between control – vehicle and control group. Quercttin treated group showed a significantly lower in blood urea and serum creatinine concentration than that of control–vehicle group (*p<0.05) as shown in Figure 1 & 2.

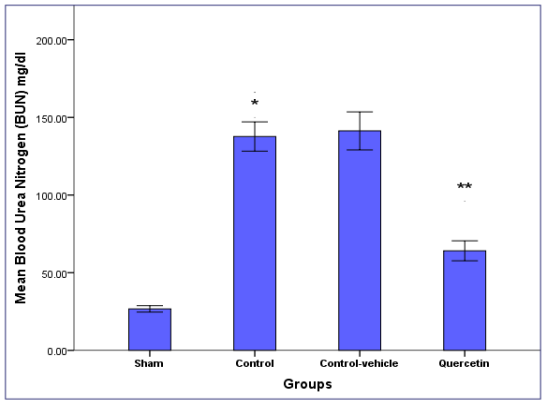


Figure (1): Error bar chart shows the difference in mean± SEM values of BUN level (mg/ml)*versus corresponding sham; versus control-vehicle, *P<0.05.**

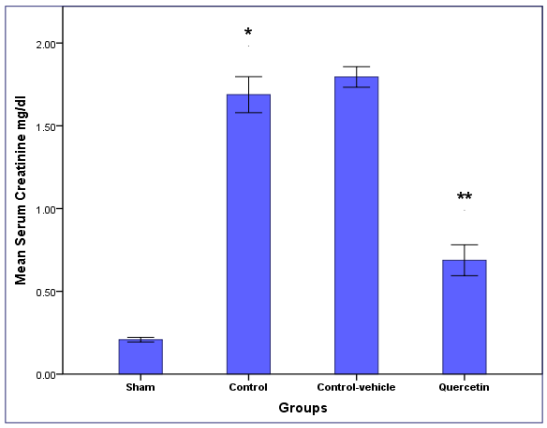


Figure (2): Error bar chart shows the difference in mean± SEM values of serum creatinine level (mg/ml)* versus corresponding sham; ** versus control-vehicle *P<0.05

Effect of Renal I/R on NGAL

The level of plasma NGAL significantly increased in control group as compared with sham group (*p < 0.05). There was insignificant difference between control – vehicle and control group. In quercttin treated group NGAL level was significantly lower than that of control–vehicle group (*p < 0.05) as shown in Figure3.

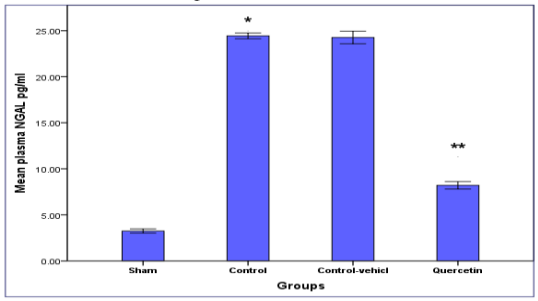


Figure (3): Error bar chart shows the difference in mean± SEM values of plasma NGAL level (mg/ml) * versus corresponding sham; ** versus control-vehicle *P<0.05

Effect of Renal I/R on inflammatory markers (IL-6 & TLR-2)

The results showed a significantly increased in IL-6 & TLR-2 concentration in control group as compared with sham group (*p < 0.05). There was insignificant difference between control–vehicle and control group. In quercttin treated group the renal IL-6 & TLR-2 levels were significantly lower than that of control–vehicle group (*p < 0.05) as shown in Figure 4 & 5.

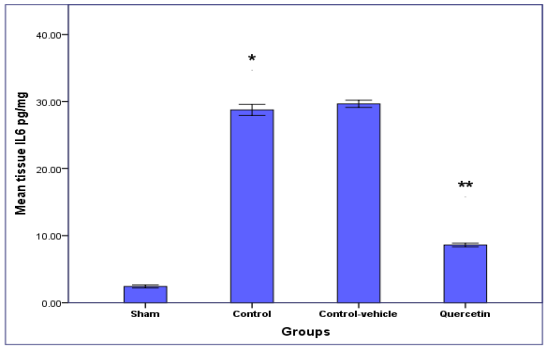


Figure (4): Error bar chart shows the difference in mean± SEM values of tissue IL-6 level (ng/mg) * versus corresponding sham; ** versus control-vehicle *P<0.05

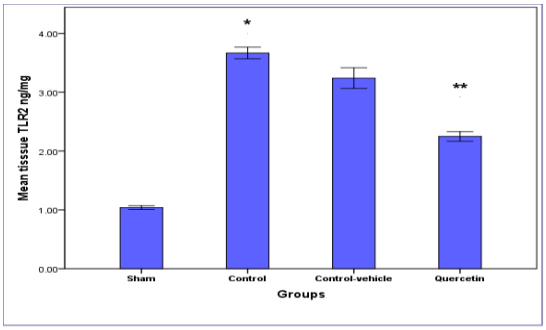


Figure (5): Error bar chart shows the difference in mean± SEM values of tissue TLR-2 level (ng/mg) * versus corresponding sham; ** versus control-vehicle,* P<0.05

Effect of Renal I/R on anti-apoptotic marker (Bcl-2)

The results showed a significantly decreased in Bcl-2 concentration in control group as compared with sham group (*p < 0.05). There was insignificant difference between control–vehicle and control, in quercttin treated group there was a significantly increased in Bcl-2 concentration when compared with control –vehicle group (*p < 0.05) as shown in Figure 6.

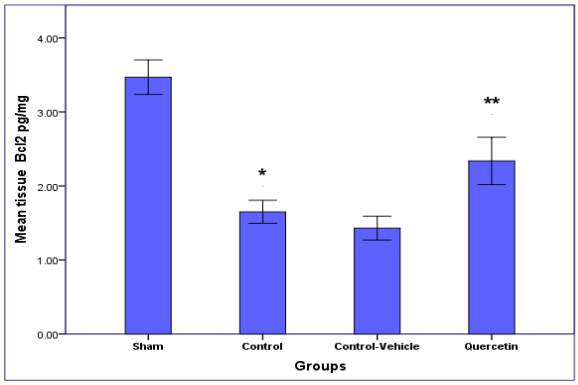


Figure (6): Error bar chart shows the difference in mean± SEM values of tissue Bcl-2 level (pg/mg) * versus corresponding sham; ** versus control-vehicle, P<0.05

Effect of Renal I/R on apoptotic marker (Bax)

The results showed a significantly increased in Bax concentration in control group as compared with sham group (*p < 0.05). There was insignificant difference between control–vehicle and control. In quercttin treated group there was a significantly decreased in Bax concentration than that of control–vehicle group (*p < 0.05) as shown in Figure 7.

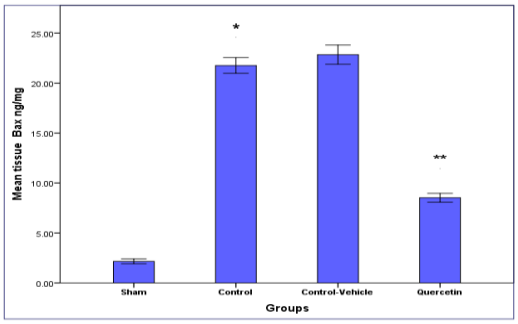


Figure (7): Error bar chart shows the difference in mean± SEM values of tissue Bax level (ng/mg) * versus corresponding sham; ** versus control-vehicle, P<0.05

Effect of Renal I/R on oxidative stress marker F2 isoprostane (F2-IsoP)

The results showed that the level of renal F2IsoP is significantly increased in control group as compared with sham group (*p < 0.05). There was insignificant difference between control–vehicle and control group. The renal F2IsoP level in quercttin treated group was significantly lower than that of control–vehicle group (*p < 0.05) as shown in figure (8).

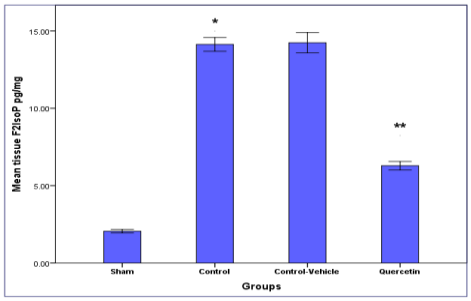
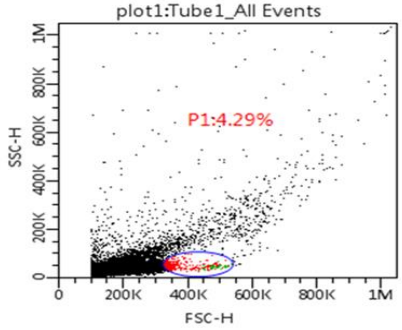


Figure (8): Error bar chart shows the difference in mean± SEM values of tissue F2IsoP level (pg/mg) * versus corresponding sham; ** versus control-vehicle, P<0.05

Effect of Renal I/R on Notch-1 Jagged-1

The level of renal Notch1 Jagge1 was measured by using flow cytometry technique, it significantly increased in control group as compared with sham group (*p < 0.05). There was insignificant difference between control–vehicle and control group. The renal Notch-1 jagged-1 level in quercttin treated group was significantly lower than that of control –vehicle group (*p < 0.05) as shown in figures (9-13).

A (scatter group)



B (histogram)

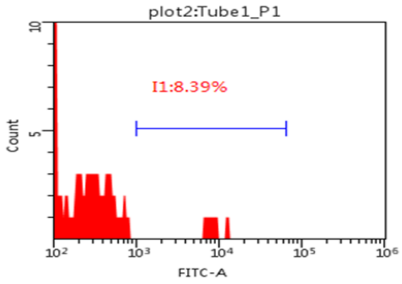


Figure (9): Representation images of flow cytometry to the percentage of renal Notch1 Jagge1 (Sham group), (A-scatter graph) & (B-histogram) showed normal percentage

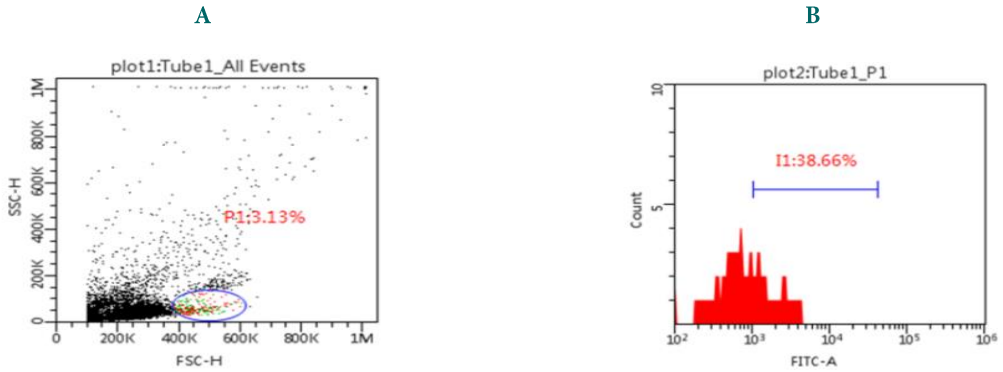


Figure (10): Representation images of flow cytometry to the percentage of renal Notch1 Jagge1 (control group), (A-scatter graph) & (B-histogram) showed high percentage

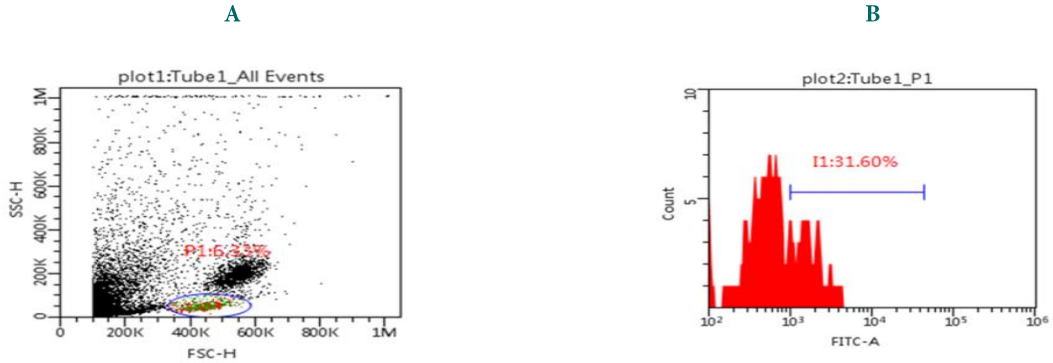


Figure (11): Representation images of flow cytometry to the percentage of renal Notch1 Jagge1 (Control-Vehicle group), (A-scatter graph) & (B-histogram) showed high percentage.

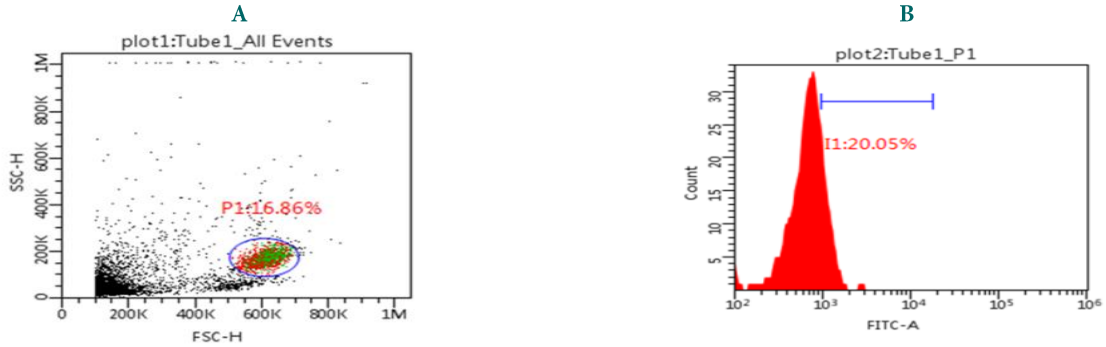


Figure (12): Representation images of flow cytometry to the percentage of renal Notch1 Jagge1 (Quercttin group), (A-scatter graph) & (B-histogram) showed lower percentage than the I/R-control group.

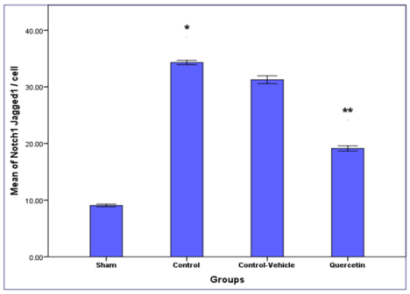


Figure (13): Error bar chart shows the difference in mean± SEM values of blood Notch-1 Jagged-1 level (%) * versus corresponding sham; ** versus control-vehicle, P<0.05

Histological findings

A cross section of sham mouse kidney showed a normal tissue appearance, There was statistically significant difference between control group and sham group (*P < 0.05) and the score of the control group showed severe renal injury (70 %) and it (30%) showed moderate injury , no statistically significant difference between control group and Control -Vehicle Group, and the score of the control -vehicle group showed severe renal injury (70%) and it (30%) showed moderate injury , treatment of mice with quercttin improved renal injury score significantly (*P < 0.05) as compared with control -vehicle group and the total score of this group were:(40%) had normal histological appearance, and (50%) of the group had slight renal injury and (10 %) had moderate injury, these changes shown in figure (14).

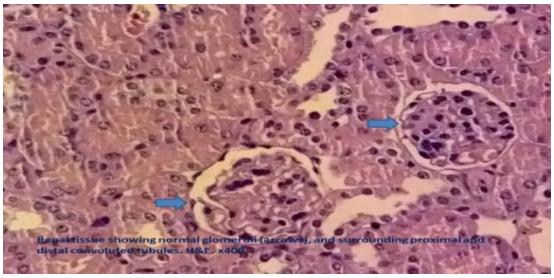
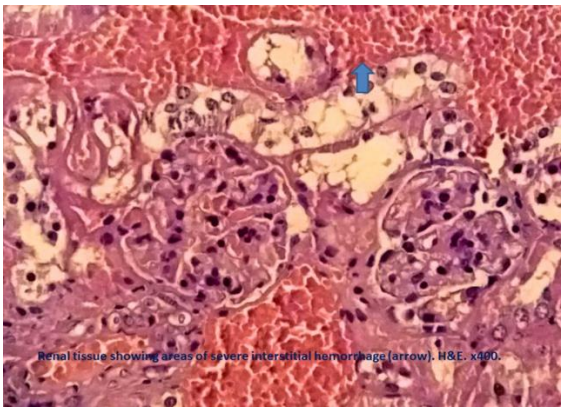
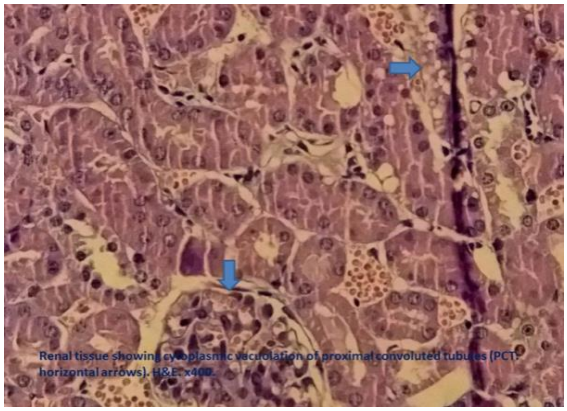


Figure (14): Section through kidney (sham group) showing normal glomeruli (arrows) and normal surrounding proximal and distal convoluted tubules . H & E stain 400X

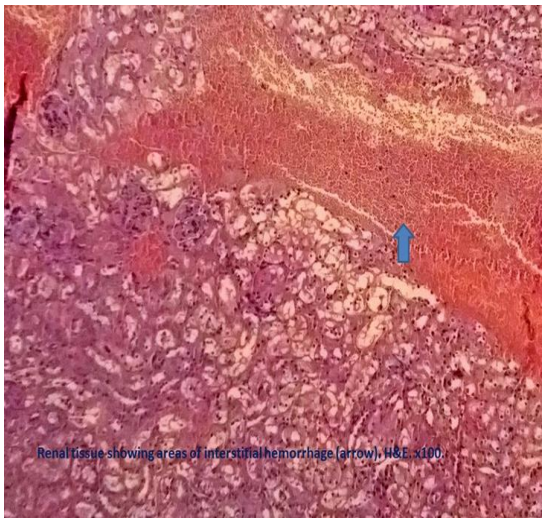


A

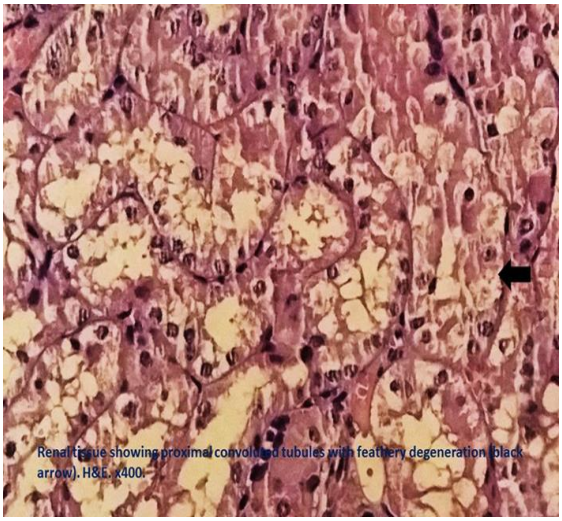


B

Figure (15): Sections through kidney (I/R-Control group) A; showing cytoplasmic vacuolation of proximal convoluted tubules B; showing areas of sever interstitial hemorrhage H & E stain 400X.



A



B

Figure (16): Sections through kidney(Control-Vehicle group) A; showing proximal convoluted tubules with feathery degeneration B; showing areas of interstitial hemorrhage H & E stain 400X

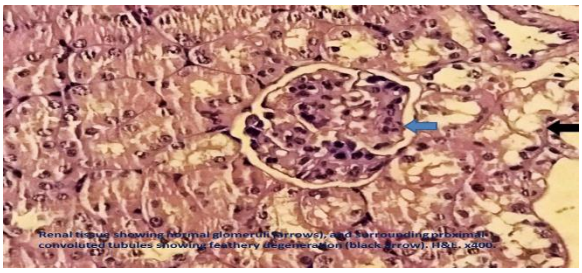


Figure (17): Sections through kidney (Quercttin group) showing normal glomeruli and feathery degeneration of convoluted tubules H & E stain 400X

Discussion

Effect of Renal IR on Renal Function Test (BUN and Serum Creatinine)

The level of BUN & Serum creatinine (S CRE) was significantly increased in the control group compared to the sham group (*P <0.05) Zou, et al [19] shown that the renal I/R-induced glomerular injury mediate podocytes effacement, cytoplasmic oedema, detachment, and even cell death and these events may explain the elevated serum level of the BUN and S CRE

Effect Of Renal I/R On NGAL

The level of plasma NGAL was significantly increased in the I/R-control group compared to the sham group (*P <0.05) Paragas et al., [20] showed that the increases in urinary and plasma NGAL are powerful predictor of AKI when compared with serum creatinine.

Effect Of Renal I/R on Inflammatory Mediators (IL-6 & TLR-2)

The renal tissue levels of IL-6 & TLR-2 were significantly increased (*P<0.05) in the control group when compared with the sham group Faubel S et al. [21] showed that the plasma IL6 increases as early as 2 hours after renal ischemia induction, elevated IL6 leads to activation and dysfunction of endothelial cells, triggering production of IL8, Neutrophils recruitment, and increased endothelial permeability. Abou-Hany HO, et al [22] showed that the activation of TLR2 by endogenous damage-associated molecular agents controls the inflammatory reactions in kidneys subjected to IRI.

Effect Of renal I/R on Apoptotic Mediators (Bax and Bcl-2)

The level of Bax was significantly increased in the control group compared to the sham group (*P <0.05) accompanied with a significant reduced Bcl-2level He, Zhiyu et al. [23] demonstrate ,in AKI model group, mRNA expression of Bax was significantly increased with an alleviated expression of Bcl-2, resulting in the apparent increase of Bax/Bcl-2 ratio.

Effect Of Renal I/R on F2 -Isoprostane

The level of F2 Isoprostane was significantly increased in the I/R-control group compared to the sham group (*P <0.05) Arulkumaran, et al [24] measured of urine F2-isoprostanes in 24-hour sham-operated and IRI animals ,measurement of F2-isoprostanes has emerged as one of the most reliable approaches to assess oxidative stress status in vivo, the levels of urine isoprostane are higher in 24-hour IRI animals compared with sham-operated animals .

The Effect of Renal I/R On Notch 1 Jagged 1

The level of Notch1 Jagged1 was significantly increased in the I/R- control group compared to the sham group (*P <0.05). Han SH., et al [25] his study shown that tubular over expression of Notch-regulated gene modulates renal damage by regulating fibrosis, mitochondrial dysfunction and lipid oxidation, suggesting that the Notch-regulated gene could be a key mediator of deleterious effects of Notch in the kidney.

The Effect of Renal I/R On kidney Parenchyma

There was statistically significant difference between control group and sham group (*P < 0.05) . The score of the control group shows sever renal injury and moderate injury may be due to elevation in tissue inflammatory cytokines that cause Leukocyte and endothelial cell interactions which plays a role in the inflammatory progression of acute renal failure and due to the relatively larger consumption of O2 in the outer medulla, this region undergoes severe vascular congestion Le Clef et al., [26] reported that the severity of histological renal damage is dependent on ischemia time (30

minutes of unilateral IRI) Causing prominent renal damage and sever loss of structure, these findings agree with the present study.

Effect of Quercttin on renal function (BUN and serum creatinine)

The use of quercttin (50 mg/kg) 30 minute before induction of ischemia caused significant lowering in serum levels of BUN and S CRE as compared with that in control group. Alhoshani et al [27] showed protective effect of Quercttin supplementation against cisplatin-induced nephrotoxicity in rats, as demonstrated by lower levels of serum creatinine and BUN as markers of kidney function.

Effect of Quercttin on NGAL

The use of Quercttin (50 mg/kg) 30 minute before induction of ischemia caused significant lowering (* $p<0.05$) in plasma levels of NGAL as compared with that in control group. These findings are consistent with a study done by Shin, Y. J., et al [28] showed that Quercttin markedly decreased the accumulation of Hg (HgCl2-induced AKI) in the kidney leading to urinary excretion of protein-based biomarkers, including neutrophil gelatinase-associated lipocalinis (NGAL).

Effect of Quercttin on Inflammatory Mediators (IL-6 &TLR-2)

The use of Quercttin (50mg/kg) 30 minute before induction of ischemia caused significant lowering (*$p<0.05$) in tissue levels of (IL-6 &TLR-2) as compared with that in control group, and that's mean Quercttin has a strong renoprotective effect against inflammation in IRI. Kempuraj, D., et al [29] confirm that the Quercttin, a natural compound able to act as an inhibitor of mast cell secretion, causes a decrease in the release of IL-6 in renal IR injury. Li, T., et al [30] showed the treatment with Quercttin significantly (*$p < 0.05$) restored the impaired expression of toll-like receptors including TLR2, These results suggest that Quercttin possessed anti-inflammatory effects, which may be attributed to its roles in suppressing the activation of TLR2, TLR4–MyD88-mediated NF-KB (Myeloid differentiation mediated Nuclear factor kappa B) signaling pathways.

Effect of Quercttin on apoptotic Markers (Bax & Bcl-2)

The use of Quercttin (50mg/kg) 30 minute before induction of ischemia caused significant lowering (*$p<0.05$) in tissue levels of Bax and significant increase in the expression of Bcl-2 as compared with that in control group. Wu, L., et al [31] pretreatment with Quercttin reduced the expression of these proapoptotic proteins and increased the expression of ant apoptosis proteins in a dose-dependent manner; we got similar results in our vitro experiments.

Effect of Quercttin on F2 Isoprostane

The results showed that the use of quercttin 30 minute before induction of ischemia and immediately at the reperfusion time caused significant lowering in tissue levels of F2 Isoprostane as compared with control group. To the best of our knowledge, there is no previous study investigate the effect of quercttin on F2 IsoP of renal ischemia–reperfusion injury in animal model and this effect may be due to antioxidant to the antioxidant effect of quercttin.

Effect of Quercttin on Notch1 Jagged1

The results showed that the mean of blood levels of Notch1 jagged 1 was significant decrease (*$p<0.05$) in Quercttin group as compared with that in control group. To the best of our knowledge, there is no previous study investigate the effect of quercttin on Notch1 Jagged1 of renal ischemia reperfusion injury in animal model and this effect may be due to the anti-inflammatory effect of quercttin.

Effect of Quercttin on Renal Parenchyma

Treatment of mice with quercttin significantly reduce renal injury (*$P< 0.05$) as compared with control group. Histological examination of the mouse kidney tissue showed changes in the renal tubules of the mouse in control group which showed swelling and cystic renal tubule dilatation with interstitial inflammation were apparent when compared with normal mouse kidney. Treatment with Quercttin to mice with ischemic injury reduced the abnormal histological renal changes. Yang, H., et al [32] showed that the improvement in renal histology in Quercttin treated group is related to the anti-inflammatory, antioxidant and ant apoptotic effects of Quercttin.

Conclusion

Quercttin possess a renoprotective effects against ischemia reperfusion by improving kidney function, it has anti-inflammatory effect as evidenced by a significant reduction in IL-6, TLR -2, NF-KB, MCP-1 and HMGB-1 and associated with Notch1 / Jagged1 inflammatory pathway in renal I/R injury, the present study further support the antioxidant effect for quercttin by the significant reduction in F2 isoprostane level. Quercttin has anti–apoptotic effect by reduce the level of the pro- apoptotic marker (Bax) and elevation to the level of anti- apoptotic marker (Bcl2)

ABBREVIATION:

Abbreviate	Word
DMSO	Dimethyl sulfoxide
IL	Interleukin
TLR	Neutrophils gelatinase-associated lipocalinis
NGAL	Toll like receptors
Bax	Bc12 associated X protein
Bcl2	Bcell lumphoma2
IRI	Ischemia reperfusion injury
ROS	Reactive oxygen species
ATP	Adenine triphosphate
I/R	Ischemia Reperfusion
TLRs	Toll-like receptors
AKI	Acute kidney injury
Dll 1/3/4	Delta it's a ligand for Notch 1-4
Jag	jagged
ELISA	Enzyme linked immunosorbent assay
H&E	Hematoxylin and Eosin
BUN	Blood Urea Nitrogen
S CRE	serum creatinine
MyD88	Myeloid differentiation
NF-KB	Nuclear factor kappa B

Units and Symbols

Physical quantity	Base unit	SI Symbol
Mass	gram	g
	kilogram	kg
	milligram	mg
	nanogram	ng
Time	hour	h
Volume	milliliter	ml

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