Potential nephroprotective effect of dapagliflozin against renal ischemia reperfusion injury in rats via activation of autophagy pathway and inhibition of inflammation, oxidative stress and apoptosis

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KEYWORDS
Renal Damage, RIRI, Dapagliflozin, Autophagy, LC-3, Akt, Anti-Inflammatory, Anti-Apoptotic, Antioxidant

ABSTRACT
Renal ischemia/reperfusion injury is a common cause of acute kidney injury (AKI), causing tissue damage, inflammation and oxidative stress. Autophagy, responsible for breakdown and recycling of cytoplasmic components. In hypoxic and ischemic renal damage, autophagy may serve as a survival strategy for the cells. Dapagliflozin has been shown to have protective effects against ischemia.
Objective: The study investigates the potential nephro-protective effects of dapagliflozin on renal ischemia reperfusion injury, evaluating its antioxidant, anti-inflammatory, anti-apoptotic effects and activation of autophagy via biochemical parameters and histopathological changes in adult male rats.
Material and method: In this study, twenty eight male rats weighing between 200-300g, were divided into 4 groups sham, RIRI, DMSO, Dapagliflozin pretreated groups. All groups subjected to 30 min. of ischemia then 24 h of reperfusion except sham undergo the same anesthesia and surgical procedures except for bilateral renal ischemia. Dapagliflozin 1mg/kg and DMSO was given 2 hours before the induction of ischemia.
Results: The study found that blood urea and serum creatinine levels increased in RIRI and DMSO groups when compared with sham group. Renal tissue levels of IL-6, Kim-1, caspase-3, LC-3 and Akt were also elevated in RIRI while GSH levels decreased in the RIRI. Dapagliflozin group showed significant increases in GSH, LC-3 and Akt levels when compared to sham group. Dapagliflozin reduced serum urea and creatinine levels, and renal tissue levels of IL-6, Kim-1, and caspase-3 also reduced.
Conclusion: Dapagliflozin significantly reduced renal damage in rat model due to its antioxidant, anti-inflammatory, anti-apoptotic, and activation autophagy.

1. Introduction

Kidneys are vital organs that maintain fluid balance, excrete waste products, and regulate blood volume. They receive one-third of the cardiac output and are located on either side of the spine. Acute kidney failure or acute kidney injury (AKI) can lead to complications such as fluid retention, anemia, heart failure, pericarditis, and electrolyte imbalance [1]. Kidneys have two major areas: the outer (cortex) and inner (medulla) regions. They filter about 200qts of fluid every 24 hours and remove waste products like urea, uric acid, and creatinine [2]. Ischemia/reperfusion injury (IRI) occurs when there is decreased blood flow to an organ, leading to hypoxia and decreased oxygen delivery to the tissues. IRI is associated with inflammation and oxidative stress response to hypoxia, which may disturb organ function [3]. IRI is the leading cause of acute kidney injury and is not uncommon in kidney transplant recipients [4] [5]. Renal IRI directly impairs renal tubules, glomeruli, blood vessels, and interstitium [6], and is a significant complication in hospitalized patients and up to 50% in ICU settings [7]. Preventive measures, such as IV fluid, avoidance of nephrotoxic drugs, regular monitoring of renal function, and correction of hyperglycemia, are essential for patients admitted to the intensive care unit [8]. Apoptosis is programmed cell death, a crucial stage in the life cycle of unwanted cells [9]. Caspases are essential markers of apoptosis and are used to test the cytotoxic effect of potential therapeutic agents [10]. Autophagy, a cellular process, controls cell degradation and recycling, and disruption in it is associated with various diseases [11] [12]. Autophagy is critical in proximal tubular cells, responsible for electrolytes reabsorption and requiring high energy. Activation of autophagy protects tubular cells from apoptosis and improves cell regeneration [13]. Protein kinase B (Akt) is involved in ischemia protective mechanisms, with some arguing activation is essential for protection against ischemia and others suggesting inhibition as a

preconditioning treatment [14] [15].

Dapagliflozin is a noval therapy for type 2 diabetes, it acts on cotransporter sodium-glucose 2 (SGLT2) by inhibition the SGLT2 in a diabetic kidney, this lead to decreases tubular oxidative stress and oxygen consumption, resulting in a net excretion of glucose in urine, it was also shown to slow down the progression of diabetic kidney disease in patients with type 2 diabetes, the exact mechanisms by which SGLT2 inhibition benefits the kidneys is not well understood yet, but one possible hypothesis is that SGLT2 inhibition reduces tubular oxygen consumption by inhibiting sodium and glucose reabsorption, thus increasing cortical oxygen availability [16].

This study investigates the potential nephro-protective effects of dapagliflozin against renal ischemia reperfusion injury through activation of the autophagy pathway.

2. Methodology

Animal preparation

The Sprague Dawley rats utilized in this study were 14–20 weeks old obtained from the University of Kufa/Faculty of Science. The rats lived in an animal house on the Faculty of Pharmacy campus, where they were kept in a controlled environment with a temperature of 24 ± 2°C and switched between light and dark cycles every day for 12 hours. They had access to drinking water and a regular meal. Prior to the commencement of the trial, two week quarantine period was instituted. The participation of the rats was permitted by the University of Kufa's Animal Care and Research Committee.

Study design

The study involved 28 male rats, divided into four groups seven rat in each group [17], sham group, RIRI group, vehicle group (DMSO + RIRI), and Dapagliflozin + RIRI pretreated group. The sham group was anesthetized and underwent bilateral flank incisions without ischemia induction. The RIRI group was anesthetized and underwent bilateral flank incisions to induce renal ischemia for 30 minutes, followed by a 24-hour reperfusion period. The vehicle group received dimethyl sulfoxide (DMSO) vehicle for dapagliflozin via oral administration 2 hours before ischemia, followed by 30 minutes of bilateral renal ischemia and reperfusion for another 24 hours [18]. The Dapagliflozin pretreated group received 1mg/kg of Dapagliflozin orally two hours before ischemia, then underwent bilateral flank incisions to induce renal ischemia for 30 min. and 24 hours of reperfusion [19]. Following 24 hour period of reperfusion, kidney and blood samples were obtained via a midline laparotomy incision. An intraperitoneal injection of anesthesia for the procedure induced by administering ketamine and xylazin at a dose of 100 mg/kg and 10 mg/kg respectively.

Drug preparation

Dapagliflozin powder dissolved in DMSO (76 mg/ml as a stock solution) as described by the manufacturers guidelines (Hyperchem for chemicales/China). The dosage was given orally based on the body weight [19].

The experimental model of renal ischemia/reperfusion injury

The procedure starts by weighing rats and anesthetizing them with intraperitoneal ketamine and xylazine. The rats are placed in a prone position over heating pads to maintain body temperature and stability. The operative site is disinfected with povidone-iodine solution. A bilateral 1.5 cm vertical flank incision is made, and a layer by layer dissection is carried out. The kidney is found below the 13th rib, and the perinephric fat is dissected to expose the renal hilum. The pedicle is skeletonized and all fat is removed to prevent incomplete renal ischemia. The renal pedicle is occluded and the timer is started to calculate the ischemia time. The kidneys are replaced into the retroperitoneal space, and renal vessels are left clamed for 30 minutes. After the ischemia time is over, the clamps are removed and reperfusion is visually confirmed. 1mL of 37°C pre-warmed 0.9% saline is given to the

retroperitoneal space before wound closure [18]. The skin wounds are closed into two layers. The rats are then moved back to their cages and provided with food. After 24 hours, the rats are anesthetized and sacrificed, and blood and kidney tissues samples are obtained for analysis.

Collection of samples

Blood samples collection for measurement of renal function

After 24 h of reperfusion, the rats are anesthetized and blood samples are aspirated directly from the heart. Approximately 2-5 mL of blood was inserted in a simple tube at 37°C with no anticoagulant. To separate the serum, the tube was centrifuged at 3000 rpm for ten minutes. The manufacturer's instructions were followed while measuring urea and creatinine levels in serum using the ELISA technique.

Tissue preparation for measurement of apoptotic, autophagy and oxidative parameters

When the experiment is over, the kidney is extracted from each animal and then sliced into two halves. One half was put into deep freeze (-80°C). And then, the frozen half was homogenized. The mixture was homogenized in an intense ultrasonic liquid processor with phosphate-buffered saline (at a weight/volume ratio of 1:10), 1% Triton X-100, and a protease inhibitor cocktail [20]. The homogenate was then centrifuged at 5000 rpm for 10 min. at 4°C, the resulting supernatant was used for the measurement of IL-6, Caspase-3, LC-3, GSH, KIM-1, and Akt using the ELISA technique according to the manufacturer's guidelines.

Preparation of tissue samples for Histopathology

Before being embedded in a paraffin block, the other kidney half was soaked in 10% Neutral-Buffered Formalin, dehydrated in alcohol, and cleaned in xylene. The staining technique employed for the horizontal slide sections, which were approximately 5µm thick, involved the application of hematoxylin and eosin stain [21].

Measurement of study parameters

Measurement of urea and creatinine

Measurement of Urea and Creatinine levels in according to the instruction supplied by the manufacturer by using Elisa Kit, from Sunlong Biotech co., Ltd. of China.

Measurement of IL-6

The IL-6 Elisa Kit, manufactured by Sunlong Biotech co., Ltd. of China, was used to measure IL-6 levels in according to the instructions supplied by the manufacturer.

Measurement of KIM

The KIM -1 Elisa Kit, manufactured by Sunlong Biotech co., Ltd. of China, was used to measure KIM-1 levels in according to the instructions supplied by the manufacturer.

Measurement of GSH

The GSH Elisa Kit, manufactured by Sunlong Biotech co., Ltd. of China, was used to measure GSH levels in according to the instructions supplied by the manufacturer.

Measurement of Caspase-3

Elisa Kit of caspase-3, manufactured by Sunlong Biotech co., Ltd. of China, was used to measure caspase levels in according to instructions supplied by the manufacturer.

Measurement-of LC-3

Sunlong Biotech co., Ltd. (China) LC-3 Elisa kits were used to measure LC-3 levels following the directions provided by the manufacturer.

Measurement-of Akt

Sunlong Biotech co., Ltd. (China) Akt Elisa kits were used to measure Akt levels following the directions provided by the manufacturer.

Statistical Analyses

The data collected in this study was analyzed using GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, California, USA). Results were shown as mean ± Standard Error Mean (SEM). One-Way Analysis of Variance (ANOVA) followed by the Bonferroni multiple comparison test were used to analyze the data. P <0.05 was used as to indicate statistical significance in all tests. Additionally, Jablonski Score system criteria was used to compare alteration in histopathology between the study groups:

3. Results and discussion

The study found that blood urea and serum creatinine levels increased in the RIRI and vehicle groups when compared with the sham group. Renal tissue levels of IL-6, Kim-1, caspase-3, LC-3 and Akt were also increased in RIRI and vehicle groups when compared with sham group. GSH levels decreased in the RIRI and vehicle groups when compared with sham group. Dapagliflozin significantly reduced serum urea and creatinine levels, and renal tissue levels of IL-6, Kim-1, and caspase-3 were significantly reduced **(Figure 1, Figure2, Figure3 figure 4, Figure 6**) respectively. Dapagliflozin pretreated groups showed significant increases in GSH, LC-3 and Akt level **(Figure 5, Figure 7, and Figure 8**). Dapaglifozin reduced the severity of kidney damage in ischemia reperfusion injury, but the RIRI group showed substantial renal damage when compared with the sham group that occur in histopathologic examination (**Figure 9**).

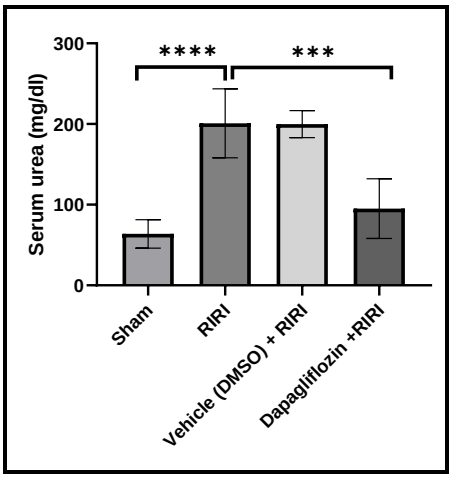

Figure 1: **Blood urea level of study groups.** Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 hr. Rats pretreated with either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The blood urea levels were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ***P< 0.001 vs RIRI, ****P< 0.0001 vs sham.

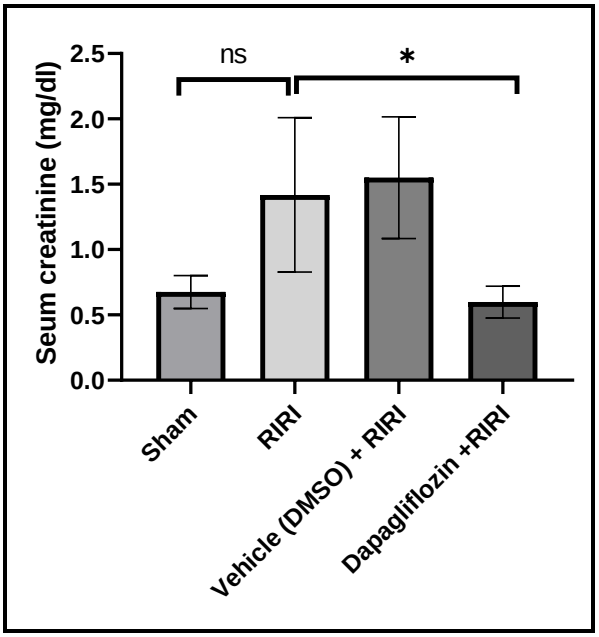

Figure 2: creatinine level of study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats were administered either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The blood creatinine levels were measured using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, *P< 0.05 vs RIRI.

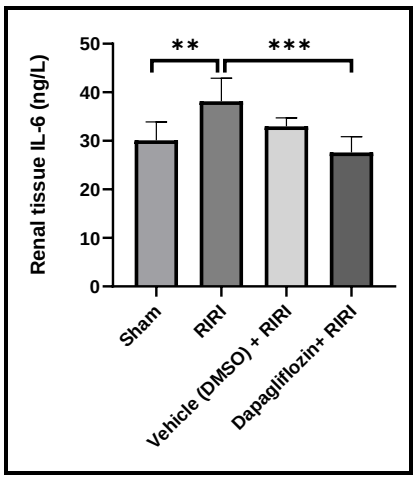

Figure 2: IL-6 level in study groups. Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats were administered with either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The tissue concentration levels of IL-6 were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, **P< 0.01 vs sham, ***P<0.001 vs RIRI

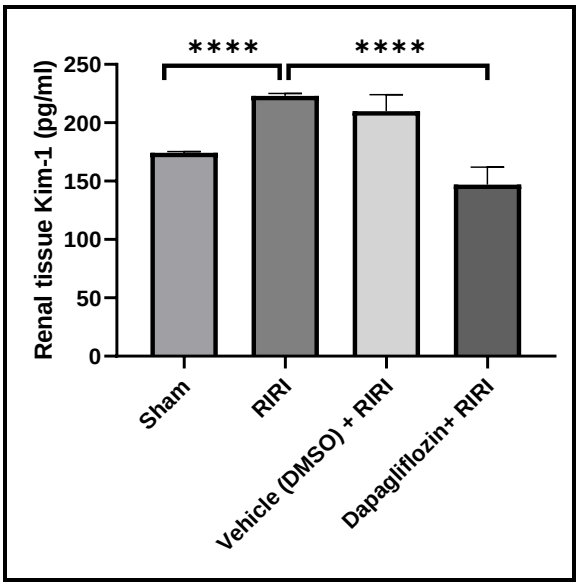

Figure 3:kim-1 level of study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats administered either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The tissue kim-1 concentration levels were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ****P< 0.0001 vs sham & vs RIRI.

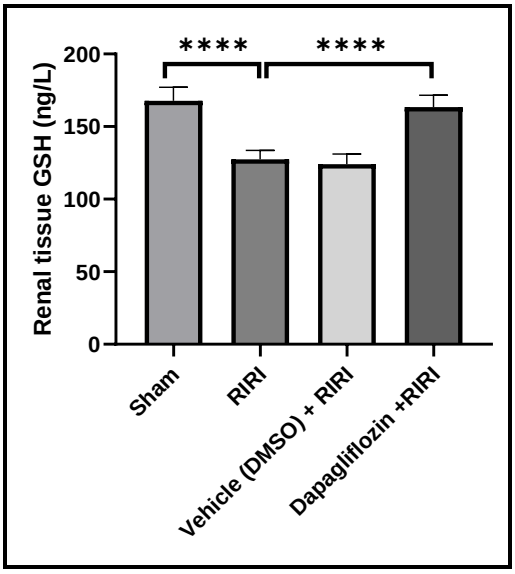

Figure 4: GSH level in study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats were administered either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). Tissue GSH concentration levels were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ****P <0.0001 vs sham and RIRI.

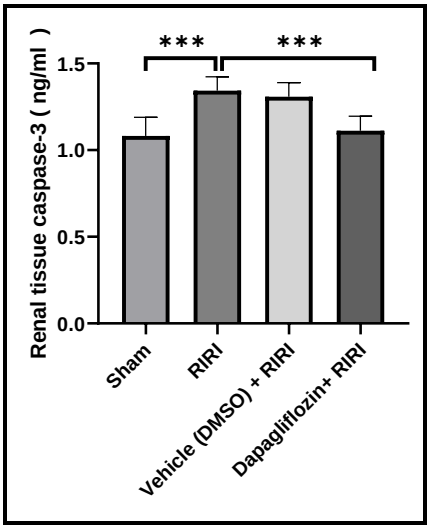

Figure 5: Caspase-3 level of study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 hr. Rats administered either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The tissue concentration levels of caspase were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ***P<0.001 vs sham and RIRI.

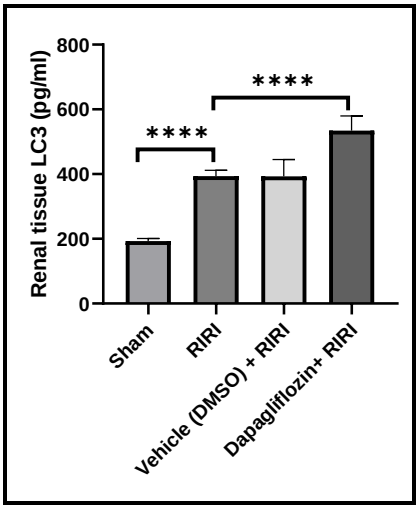

Figure 6: LC3 level of study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats administered either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The tissue concentrations levels of LC-3 were measured by using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ****P< 0.0001 vs sham and RIRI.

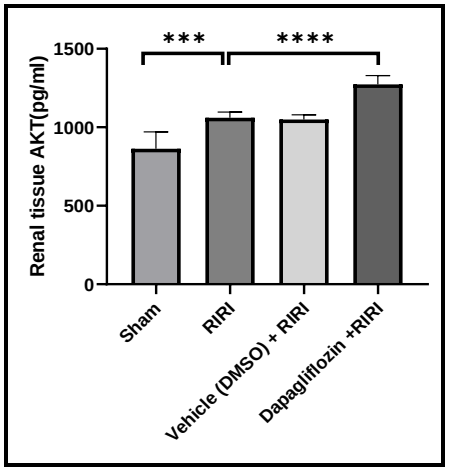

Figure 7:Akt level of study groups.

Rats of RIRI group were exposed to 30 min. of ischemia then reperfusion for 24 h. Rats administered with either DMSO, Dapagliflozin (1mg/kg), or remained without treatment (sham and RIRI). The tissue concentration levels of AKT were measured by Using ELISA technique. One-way ANOVA followed by the Bonferroni comparison test was used for analysis. Data are presented as mean ± SEM, n=7 rats in each group, ***P <0.001 vs sham and ****P <0.0001 vs RIRI.

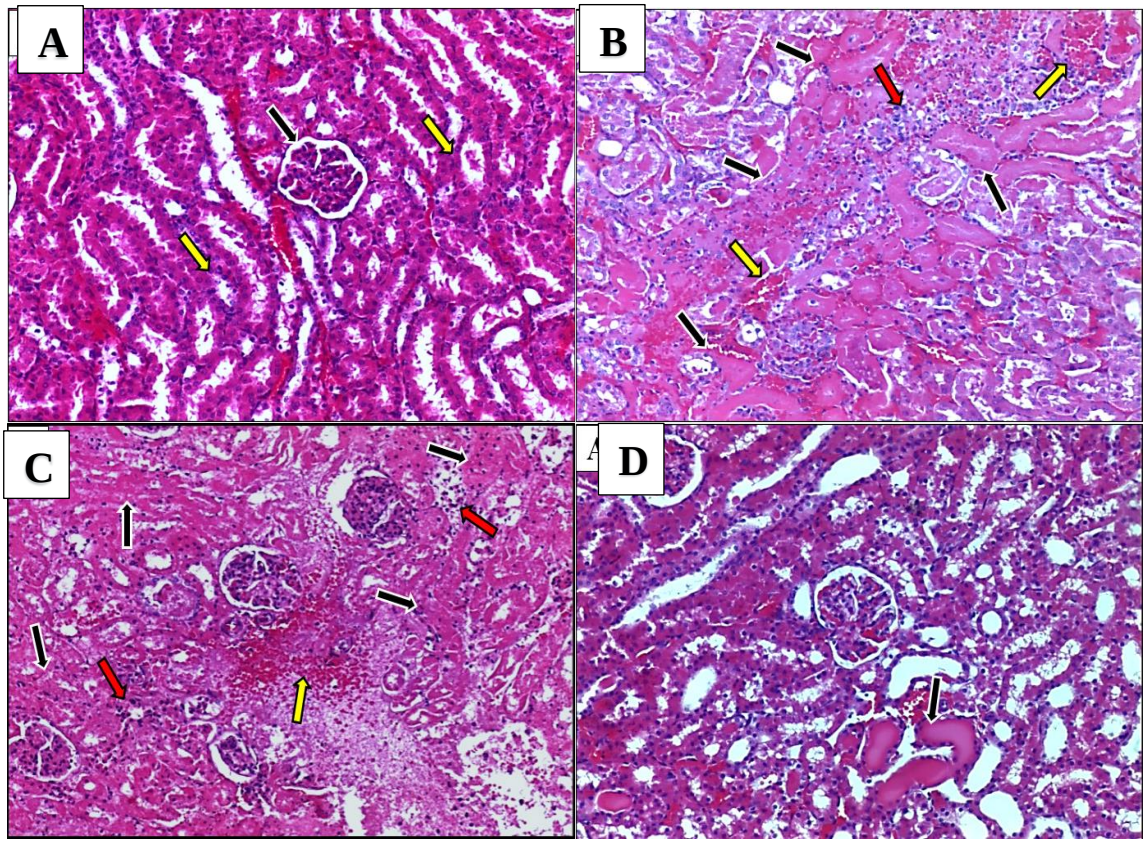

Figure 9: (A) Sham group, Normal histological architectures of kidney cortex, note the glomerulus (black arrow) and renal convoluted tubules (yellow arrow). H&E. 100x. **(B)** RIRI groups, the massive severe coagulative necrosis (black arrow) that involved all affected cortex areas led to the loss of normal cortex architectures was observed, with the presence of inflammatory cell infiltrations (red arrow) in the affected area. Coagulative necrosis was observed as the absence of the nucleus of renal tubules epithelial cells in most renal tubules of cortex areas. Also, the hemorrhage (yellow arrow)

was observed in the cortex-affected areas. H&E. 100x. **(C)** DMSO groups, the massive severe coagulative necrosis (black arrow) that involved all affected cortex areas led to the loss of normal cortex architectures was observed, with the presence of inflammatory cell infiltrations (red arrow) in the affected area. Coagulative necrosis was observed as the absence of the nucleus of renal tubules epithelial cells in most renal tubules of cortex areas. Also, the hemorrhage (yellow arrow) was observed in the cortex-affected area. H&E. 100x. **(D)** Dapagliflozin pretreated group, the coagulative necrosis (black arrow) of convoluted renal tubules epithelial cells was observed in affected cortex areas, however, the coagulative necrosis was observed in individuals of convoluted renal tubules in cortex areas, where coagulative necrosis involved about 5% of the cortex area. H&E.100x.

4. Discussion

Rapid decline in renal function, marked by elevation of blood creatinine and diminution in urine output, is known as acute kidney injury. Hypoxia, ischemia/reperfusion damage, inflammation, oxidative stress, medications, and sepsis are the primary factors that lead to AKI. Rapid energy loss, reduced membrane potential, loss of ionic hemostasis, and cell death are all mitochondrial processes that contribute to the complicated process of ischemia/reperfusion (IR) damage. Experimental RIRI has been tested with antioxidant and anti-inflammatory medications such as dapagliflozin while the studies has demonstrated a significant increase in the levels of urea and creatinine in the RIRI and vehicle groups in relation to the sham group [22]. Blood urea nitrogen and creatinine concentrations were significantly reduced when dapagliflozin was administered prior to ischemia induction **(Figure1, Figure2),** indicating a reno-protective effect from IR damage, according to the research. Earlier research in mice models of ischemia and 24 hours reperfusion confirmed a decrease in blood urea nitrogen, confirming this finding [23].

Both the RIRI and vehicle groups showed an upregulation of inflammatory markers such IL-6 and KIM when contrasted with the sham group **(Figure3, Figure 4).** According to these results, dapagliflozin, when administered orally 2 hour prior to ischemia induction, can reduce the inflammatory response and enhance renal function this finding also in agreement with the study of zhu et al., that reported in their study the protective effect of dapagliflozin on the kidneys in cases of renal ischemia/reperfusion injury by proving that it significantly reduced the inflammatory markers including IL-6 and KIM-1 when given once daily by oral gavage before ischemia induction [19].

By reducing the stress on the proximal tubular epithelium and hypoxia-induced damage, dapagliflozin can lower renal KIM-1 levels in diabetic mice, according to studies. Previous research has shown that dapagliflozin have anti-inflammatory effects, which are supported this study [24].

A significant drop in GSH levels was another consequence of renal ischemia reperfusion, suggesting that the kidney's ability to fight free radicals was diminished. Previous research has shown that rats subjected to bilateral renal ischemia had considerably lower GSH levels than the sham group (**Figure 5**) [25] [26],

This study examined that dapagliflozin is a potent medication that can effectively decrease oxidative stress and enhance antioxidant activity by elevating GSH levels. Increased levels of caspase-3, a renal marker of apoptosis essential for DNA fragmentation and cell death, were seen after renal ischemia/reperfusion damage [27] [28]. In contrast, dapagliflozin decreased caspase-3 levels in rats that were pretreated with it, indicating that it may be able to decrease apoptosis in renal tissue following IRI. Dapagliflozin may have an antiapoptotic impact, according to this study **(Figure 6**).

Levels of autophagy indicators, such as LC-3 and Akt, were significantly elevated in rats that exposed to 30 min. of ischemia and then 24 h of reperfusion, according to this study (**Figure 7, Figure 8).** These findings corroborate prior research showing that renal IRI acted as a renoprotective and encouraged autophagy activation. These results were similar to the findings of Zhang et al, renal ischemia/reperfusion in rats and found that the LC-3 levels were significantly increase in the I/R

group, concluding that renal IRI promoted the activation of the autophagy and that it had a renoprotective role [29]

This research looks at how the rat medications dapagliflozin affect indicators of autophagy. When given two hours before ischemia, dapagliflozin significantly raises LC-3 and Akt levels in comparison to the sham group. This agrees with what has been shown in other study about dapagliflozin protective function in RI. Zhu et al., showed that dapagliflozin exerted a renal protective role in rats exposure to renal IR leads to the activation of the Akt signaling pathway, thus promoting autophagy when given once daily by oral gavage before induction of ischemia if compared to the I/R and vehicle groups [19]. These results are similar to the findings of Zhang et al., who studied the action of dapagliflozin in the management of diabetic nephropathy in rats, there was an increase in the expression of LC-3, thus proving its effect in promoting autophagy [30]. Sham group demonstrated normal levels of renal parenchyma. On the other hand, significant changes were seen in the RIRI and vehicle groups on histological examination, such as coagulative necrosis, abnormal architectural loss, inflammatory cell infiltration, and considerable bleeding. When compared to the RIRI group, dapagliflozin considerably decreaseded the severity of renal damage (**Figure 9).** These finding are similar to the results obtained by who studied the beneficial effects of dapagliflozin when given to mice with adenine induced renal injury, as it showed dapagliflozin alleviated the tubulointerstitial injury and interstitial ECM deposition [31]. Another study by Chang and associates, evaluated the effect of dapagliflozin in mice with ischemia renal injury, According to their research, the use of dapagliflozin reduced the severity of damage to the renal tubules [23].

4. Conclusion and future scope

This study showed that dapagliflozin dramatically reduced renal damage from ischemia reperfusion injury in male rat model through its antioxidant effect by elevation antioxidant marker GSH, anti-inflammatory by reducing inflammatory markers IL-6 and KIM-1, anti-apoptotic by decreasing apoptotic marker Caspase-3, activation autopaghy by increasing LC-3, Akt, and ameliorating histopathological changes.

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