ORIGINAL ARTICLE

Evaluation of the nephroprotective effect of Dibenzazepin γ-secretase inhibitor on renal ischemia reperfusion injury in male rats

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ABSTRACT

Aim: This study was performed to investigate the Dibenzazepin potential nephroprotective effect on bilateral renal ischemia/reperfusion injury in model of male rats.

Materials and Methods: Male rats (number 40) were classified into four groups' n=10: first sham, second control, third DMSO, and fourth DBZ, the sham group had a median laparotomy under anaesthesia without induction of ischemia/reperfusion; the control group underwent clamping for thirty minutes on the bilateral renal artery, after that two hours of reperfusion; the vehicle group received DMSO one hour before induction of ischemia; and the DBZ group received 2 mg/kg of DBZ one hour before ischemia. Biochemical parameters (Kidney injury molecules KIM1, IL-1β, TNF-α, F2-isoprostane, GSH, and caspase-3) were assessed by ELISA technique. Furthermore, histological changes were investigated, and the Notch signalling pathway was evaluated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Results: IRI caused a significant increase in renal tissues of kidney injury molecules (KIM1), IL-1β, TNF-α, F2-isoprostane, and caspase-3) with significant decreased the level of GSH, DBZ pre-treated mitigated these effects by significant enhancing antioxidant markers and decreased inflammatory and apoptotic markers. Improving histological results and significant decrease the Notch1 and Jagged-1 gene expression in kidney tissues after renal ischemia/reperfusion damage.

Conclusions: DBZ (γ-secretase inhibitor) has considerable nephroprotective benefits in renal IRI *via* inhibiting the Notch pathway, anti-apoptotic, antioxidant, and anti-inflammatory effect.

KEY WORDS: renal IRI, DBZ, inflammation, oxidative stress, Notch1, Jagged-1, apoptosis, nephroprotection

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INTRODUCTION

Kidney ischemia-reperfusion (IR) damage present when there is a brief decrease in blood flow to the kidneys, which is return comes after that. Cellular stress is the result of oxygen and nutrient depletion caused by this disruption in blood flow. Restored of blood flow caused triggered of burst from ROS. This increases inflammation and oxidative stress, which cause damage of renal tubules and lead to acute kidney injury (AKI). Significant alterations take place in the mitochondria under certain circumstances, such as decreased mitochondrial energy, excessive mitochondrial fragmentation, and increased formation of (ROS) within the mitochondria [1]. The following of ischemic insult, a significant decrease in mitochondrial adenosine triphosphate generation, consequent opening of mitochondrial permeability transition pore and membrane potential mPTP [2]. Proximal tubule cells' ATP levels drop to a trough in a minutes after ischemia is induced [3]. Moreover, this fall in the levels of ATP might limit Na+/K+-ATPase activity, resulting in the build-up of intracellular Na+ and the subsequent influx of Ca2+, which leads to mitochondrial aggregation. After reperfusion, significant enhancements in reactive oxygen species and calcium ions levels inside mitochondria might cause dysfunction of mitochondrial [3-4]. Reactive oxygen species such as the hydroxyl radical interact with DNA cause damage the ribose-phosphate backbone. Additionally, reactive oxygen species' interactions with lipid bilayers remove hydrogen atoms from unsaturated fatty acids which bound to phospholipid; this process is called lipid peroxidation which lead to reduce the integrity and functional of cellular membranes [5-6]. Consequently, that cause the mPTP to release cytochrome C, starting a caspase cascade and beginning the mitochondrial death pathway [7-8]. Notch signalling pathway; Mammals contain Notch ligands include Jagged-1, Jagged-2, Delta-like 1 , Delta-like 3 , and Delta-like 4, as well as 4

Notch receptors (from Notch one to 4). The receptor and its ligands are represent transmembrane proteins with many extracellular domains [9]. Ligand interaction induces a modulation in the receptor which cause stimulating two successive proteolytic processes. The extracellular area is shedding as a result of the primary cleavage, that is achieved by metalloproteases belonging to the ADAM own family. The γ-secretase complex catalyses the second one cleavage, which occurs within the transmembrane domain. Following that, the Notch intracellular domain (NICD) is cleaved and travels to the nucleus to form a transactivation complex [10]. This technique removes co-repressing complexes via co-activators, leading in the transcription of Notch target genes which compose two families of transcriptional factors include hairy/enhancer-of-split linked with YRP-Wmotif (Hey) (HEY1 and HEY2), and hairy-enhancer of split (Hes) (HES-1 and HES-5) [11]. Moreover, at the time of ischemia reperfusion the activation of Notch pathway by ROS lead to activate metalloproteases (ADAM17) so indirectly trigger the discharge NICD, NICD translocate to the nuclease and activate transcription of its target gens which cause the formation of fibrosis [12]. Dibenzazepin (DBZ) is a γ-secretase preventing the activation of all Notch signalling pathway by inhibit the Notch receptors [13]. Several γ-secretase inhibitors exhibit anti-proliferative and anti-inflammatory effects [14-15]. DBZ contributes to protecting mechanisms through its anti-inflammatory and antioxidative consequences, restoring regular levels of GSH, CAT, MDA, and iNOS to mitigate oxidative damage. Additionally, DBZ exhibits anti-tumour activity towards various types of cancer cells, beside it action on Notch pathway [16-17].

AIM

This study was performed to investigate the Dibenzazepin potential nephroprotective effect on bilateral renal ischemia/reperfusion injury in model of male rats.

MATERIALS AND METHODS

PREPARATION OF ANIMALS

We got 40 male Wistar albino rats aged 8 to 12 weeks from the University of Kufa's Faculty of Science, weighing between 200 and 300 g each. The rats lived at the Faculty of Science's animal facility at Kufa University. The animals were kept in cages with a 12h light/dark cycle, a temperature of 22 ± 2°C, and humidity levels ranging from sixty to sixty five percent. The rats fed a standard diet of commercial regular chow pellets and tab water. The Institutional Animal Care were used in all experimental protocols and Use Committee (IACUC) at the University of Kufa.

STUDY DESIGN

Forty Wistar albino male rats were randomly divided to four groups of ten each: sham, control, DMSO, and DBZ. The sham group had a median laparotomy under anaesthesia with no ischemia or reperfusion in the kidneys. While the IRI group (control group) submitted to clamping in bilateral renal artery for thirty minutes, after that two hours of reperfusion, the vehicle group received the DMSO 1 hour intraperitoneally one hour before the induction of ischemia [18-19]. In the DBZ group, 2 mg/kg DBZ was given intraperitoneally one hour before the start of I/R [20] and for anaesthesia during the surgery, the rats received IP injection of 10 mg per kg of xylazine and 100 mg per kg of ketamine [21].

ETHICAL CONSIDERATIONS

All animals that involved in this study deal with care, also in handling, treatment; feeding, scarification, and surgery operation criteria according to Institutional Animal Care and Use Committee (IACUC) at Kufa University by taking the approval after complete the required experiences or instructions (approval number 20546 in 29/8/2024).

SAMPLING TECHNIQUES

TISSUE SAMPLING FOR BIOCHEMICAL ANALYSIS

The tissues of kidney were kept at -80°C until homogenized using a high-intensity ultrasound liquid processor in 1:10 W/V phosphate buffered saline containing 1% Triton X-100 and a protease inhibitor cocktail. The homogenate samples were centrifuged at 4°C for 15 minutes at 17,550 × g [22-23], then we tested the supernatants for kidney damage molecules (KIM, TNF-α, IL-1β, F2-isoprostane, GSH, and caspase-3).

TISSUE SAMPLING FOR HISTOPATHOLOGY

The tissue of kidney slice was fixed in ten percent formaldehyde, then dehydrated with series of alcohol, rinsed with xylene, and embedded in paraffin. Kidney tissues were paraffin-fixed and cut into 5-m-thick slices. The sections were then stained with hematoxylin and eosin. Damaged cells were identified and scored in five non-overlapping pictures. The scoring system

Table 1: Primers of gene expression experiment

Host	Gene		5'-3'	Product (bp)	Accession number	Reference
Rattus	Jagged1	F	AACTGGTACCGGTGCGAA	190	XM_032904296.1	[24]
		R	TGATGCAAGATCTCCCTGAAAC			
Rattus	Notch1	F	CACCCATGACCACTACCCAGTT	186	XM_032903023.1	[24]
		R	CCTCGGACCAATCAGAGATGTT			
Rattus	GAPDH	F	ATGACTCTACCCACGGCAAG	89	NM_017008	[25]

Source: Own materials

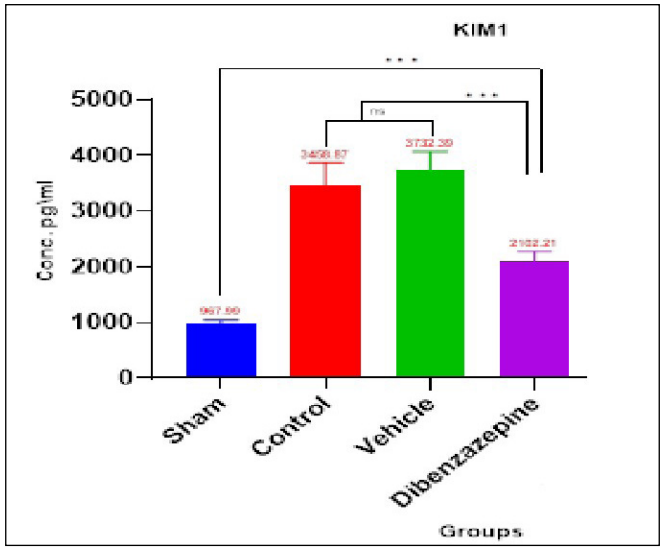

Fig. 1. Impact of DBZ on KIM1 levels following renal IR, tissue KIMI (pg/ml) among groups
Mean ± SD, n=10; ***P≤0.001 vs. sham; ***P≤0.001 vs. control/vehicle
Source: Own materials

used five scores: 0 for normal kidney tissue, 1 for kidney damage area less than 25%, 2 for kidney damage area between 25% and 50%, 3 for kidney damage area 50%-75%, and 4 for kidney damage area higher than 75%.

ANALYSIS OF NOTCH SIGNALLING PATHWAY BY QRT-PCR

Notch-1 and Jagged-1 expression levels in the kidney were measured using qRT-PCR. For complete RNA extraction, we consumed around 50-100 mg of kidney tissue precisely following the Easy-spin™ (DNA free) total RNA extraction Kit (Intron/Korea) adhere to the manufacturer's protocol, cDNA was created using reverse transcription using Add Script cDNA Synthesis Kit following the manufacturer's protocol. PCR was performed according to the instruction of GoTaq® RT-qPCR System. The sequences of the primer show in table 1 for specific gene amplification. The 2-ΔΔCt technique

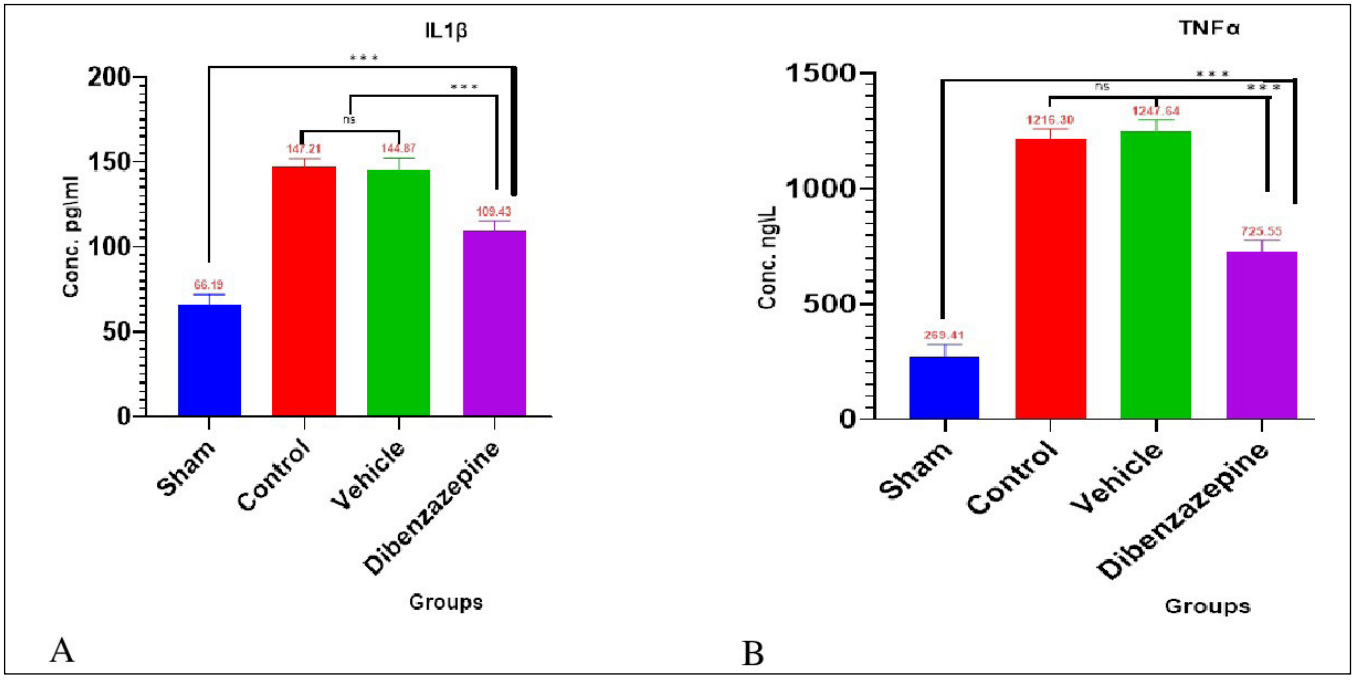

Fig 2. Effect of DBZ on TNF-α and IL-1β levels after renal IR, including tissue TNF-α (ng/L) and IL-1β (pg/ml) between groups, n = 10; mean ± SD.
***P≤0.001 *vs.* sham ****P≤0.001 compared control/vehicle*
Source: Own materials

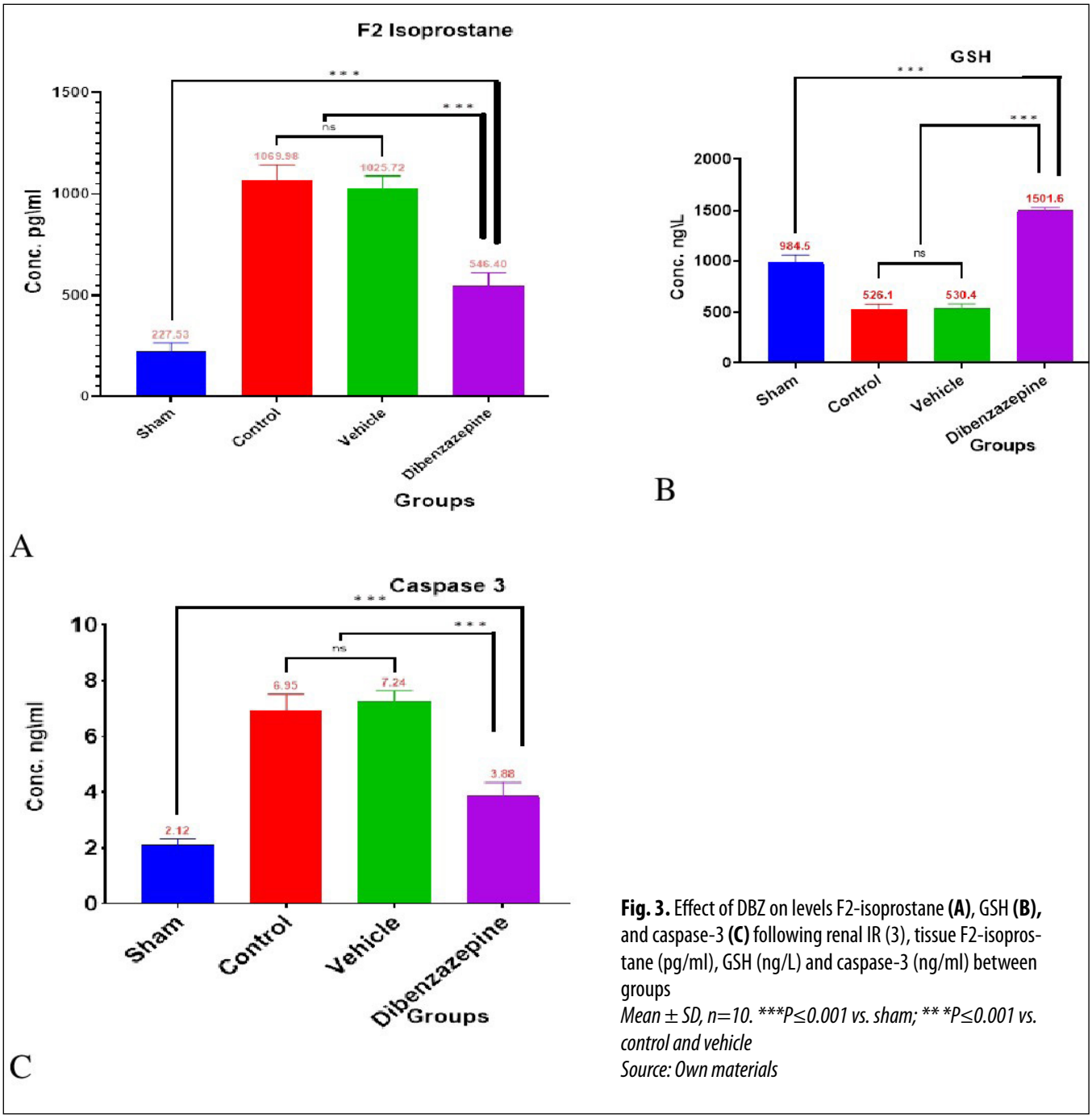

Fig. 3. Effect of DBZ on levels F2-isoprostane **(A)**, GSH **(B),** and caspase-3 **(C)** following renal IR (3), tissue F2-isoprostane (pg/ml), GSH (ng/L) and caspase-3 (ng/ml) between groups

*Mean ± SD, n=10. ***P≤0.001 vs. sham; ** *P≤0.001 vs. control and vehicle*

Source: Own materials

was used to determine fold changes in gene expression. Gene expression is quantified as a relative fold change to GADPH, an internal control reference gene.

INVESTIGATIONS

BIOCHEMICAL MARKERS ANALYSIS

By using Elisa kits obtained from SunLong Biotech Co.LTD, China, measured the levels of [KIM1 (Catalogue Number: SL0433Ra), IL-1β (Catalogue Number: SL0402Ra), and TNF-α (Catalogue Number: SL0722Ra), F2-isoprostane (Catalogue Number: SLD2059Ra), GSH (Catalogue Number: SL1093Ra), and caspase-3 (Catalogue Number: SL1366Ra)].

STATISTICAL ANALYSIS

Statistical analysis for this study was done by using Graph Pad Prism version 8 software. After testing the normal distribution of data .The one-way ANOVA test used to parametric variables and selected Post Hoc. Test using Bonferroni method to make multiple comparisons between groups. Statistical significance in all the tests was considered when $P \leq 0.001$. All data are expressed as mean ± SD.

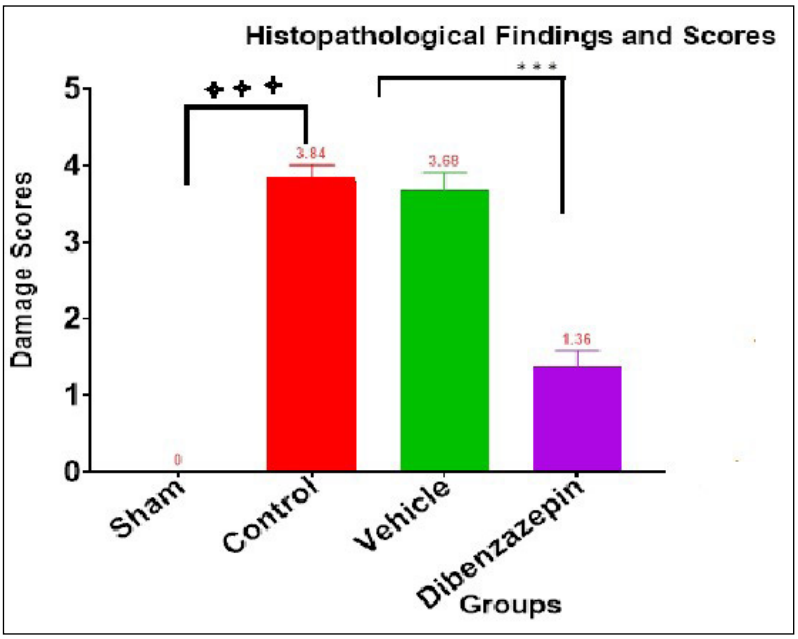

Fig. 4. Mean of Histopathological kidney injury score
+++p < 0.001 *vs*. sham group
****p < 0.001 vs. control & vehicle groups, the Kruskal-Wallis test was used for analysis, data are expressed as mean ± SD*
Source: Own materials

RESULTS

The ischemia lasted 30 minutes, followed by two hours of reperfusion. Prior to one hour of ischemia, the rats were given DMSO, DBZ (2 mg/kg), or left untreated (Sham and control groups). Several biochemical indications were employed to determine the severity of renal damage.

IMPACT ON KIDNEY INJURY MOLECULE (KIM1 1)

Following renal I/R, the IRI (control) group had a significantly higher tissue level of KIM1 than the Sham group. The amount of KIM 1 in tissues dropped considerably following DBZ therapy (Fig. 1).

IMPACT ON KIDNEY TISSUE INFLAMMATORY MARKERS

The IRI group showed significantly higher levels of IL-1β and TNF-α in their tissues than the sham group. The DBZ group dramatically reduced tissue inflammatory parameters (TNF-α and IL-1β) (Fig. 2).

IMPACT ON OXIDATIVE AND APOPTOTIC MARKERS

F2-isoprostane, and caspase-3 levels in rat renal tissue were significantly more in IRI group than in the Sham group while GSH level in rat renal tissue were significantly lower in the IRI group than in the Sham group. DBZ therapy significantly enhanced antioxidant marker (GSH) levels in renal tissue while significantly decreasing apoptotic marker (Caspase-3) levels and oxidative marker F2-isoprostane in comparison to the control (IRI) group (Fig. 3A-C).

HISTOPATHOLOGICAL FINDINGS

Sham group reported much less damage as compared with other groups. DBZ group exhibited much lower damage than the control and DMSO groups, suggesting a nephroprotective effect (Fig. 4).

The control group showed a marked abnormality in renal structure with severe renal changing of tissue architecture including tubular increased cytoplasmic eosinophilia, cellular swelling, degeneration of tubular epithelium, vascular congestion, eosinophilic cast and cytoplasmic vacuoles (score = 4 and represent > 75% of damage). The group received DMSO had similar pathology to the IRI group. The DBZ group which observed moderate changes in renal architecture (score = 2), figure (5). The Sham group had have a normal histological appearance without morphological change. The control group had severe damage in renal tubules, characterized by Cytoplasmic swelling and increased cytoplasmic eosinophilia, cytoplasmic vacuoles, and eosinophilic cast. The DMSO group had the same histopathological changes to control group. DBZ pre-treated group the renal tubules damage with score 2 characterized by involving 30% of the examined damaged tubules (Fig. 5A-F).

IMPACT ON NOTCH1 AND JAGGED1 M RNA EXPRESSION

The renal tissue levels of Notch1 and Jagged-1 expression in vehicle and control groups were significantly ($p<0.001$) more than that in the sham group, while the vehicle group was no insignificant difference than control group. On the other hand, the level in the Dibenzazepin was significantly lower ($p<0.001$)

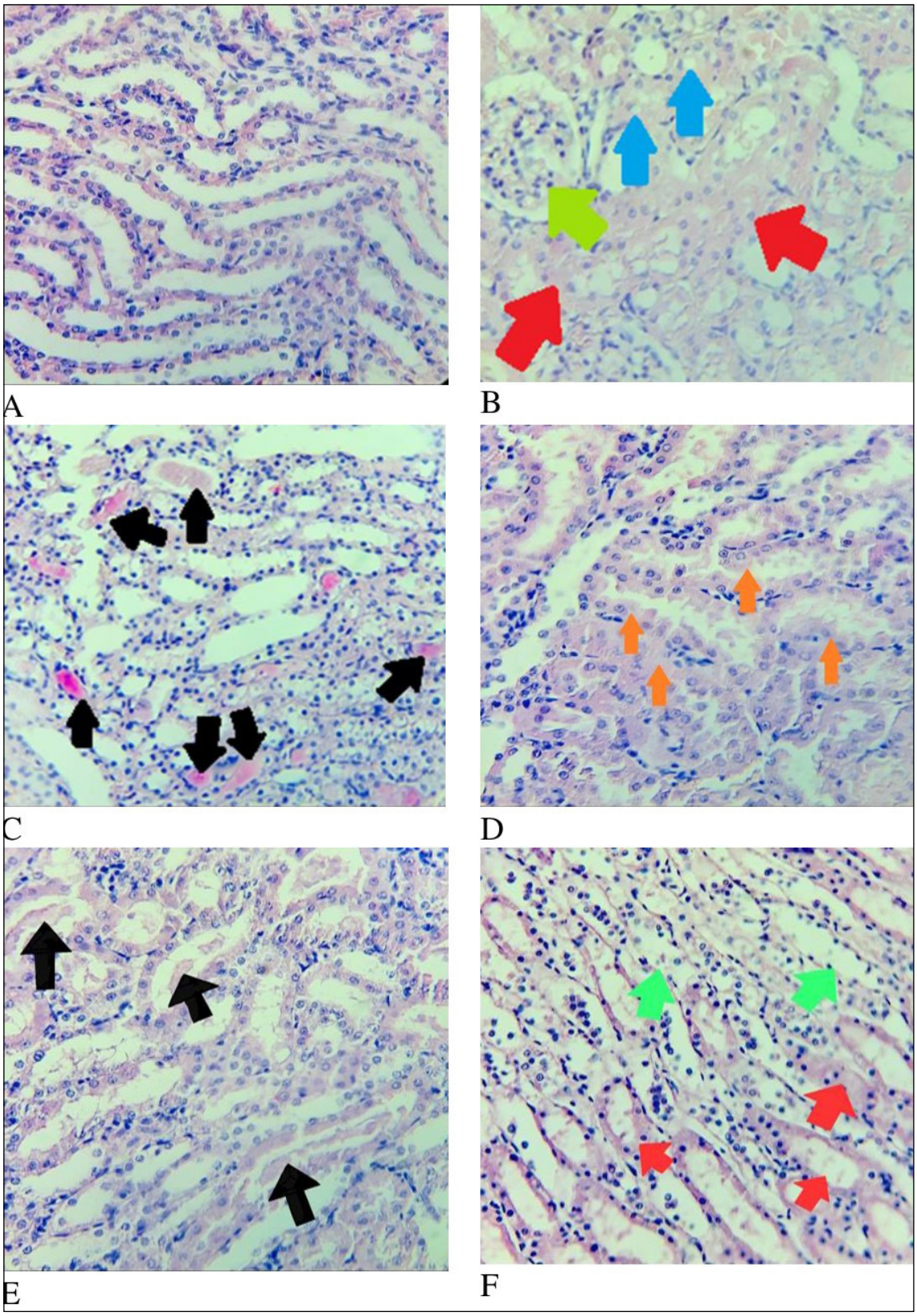

Fig. 5. Histopathological analysis of renal tissues, **(A)** Sham group has normal renal tubules, **(B) & (C)** Control group, renal tubules with score 4 damage, cytoplasmic swelling and increased cytoplasmic eosinophilia (red arrows), cytoplasmic vacuoles (blue arrows), eosinophilic cast (black arrows), normal glomerulus (green arrow); **(D) &(E)** Vehicle group, renal tubules with score 4 damage, Cytoplasmic swelling and increased cytoplasmic eosinophilia (red arrows), eosinophilic cast (black arrows), vascular congestion (orange arrow); **(F)** DBZ group renal tubules with score 2 damage, damaged tubules (red arrows), normal tubules (green arrows), H&E. X400
Source: Own materials

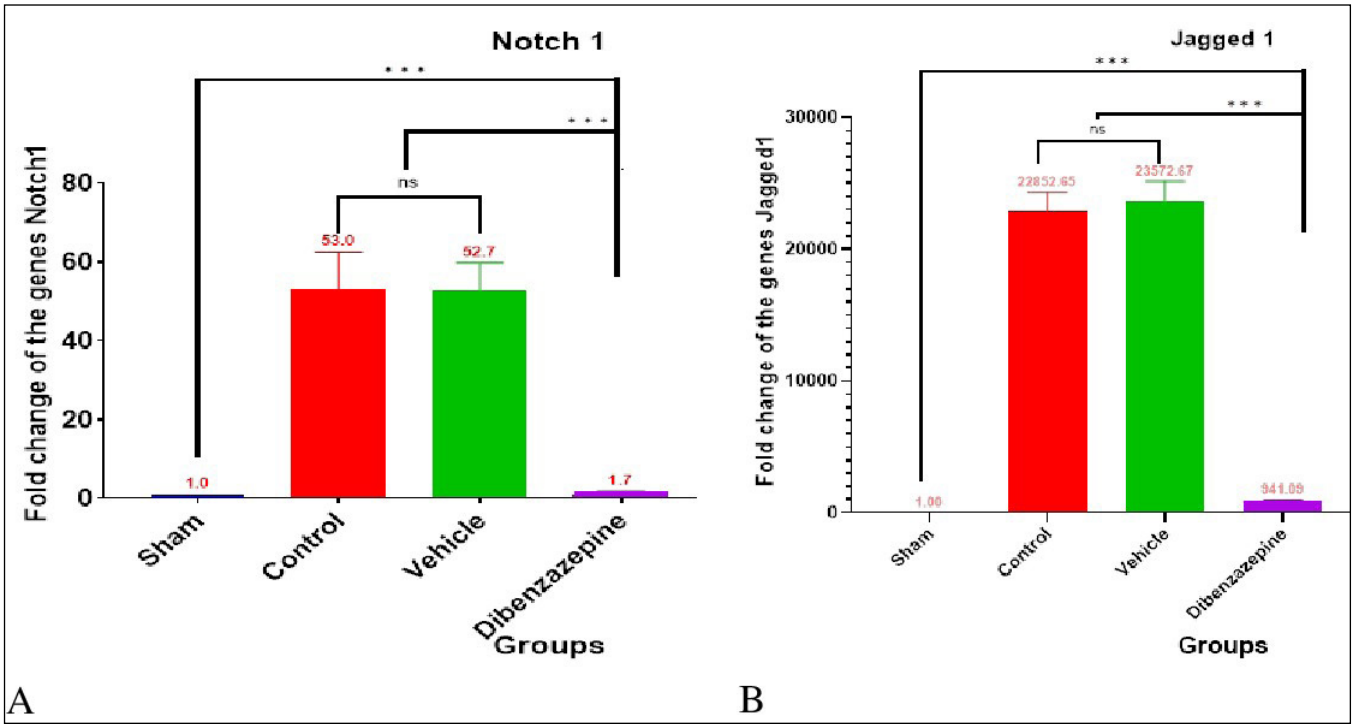

Fig. 6. (A) The bar graph Notch gene expression, **(B)** The bar graph Jagged-1 gene expression, all data were expressed as mean ± SD (n = 10). Sham group *vs.* vehicle & control groups
****P < 0.001; DBZ treated group vs. vehicle & control groups, ***P. < 0.001*
Source: Own materials

than that in the sham, control, and vehicle groups (Fig. 6A-B).

DISCUSSION

Ischemia is a rapid temporary reduction in blood supply to the organ and subsequent reoxygenation which accompanied by a strong oxidative stress and inflammatory response to hypoxia and reperfusion lead to disrupts organ function [26] due to exacerbation in tubular and glomerular damage, and producing a strong inflammatory response, called "reperfusion injury" [1]. This research aims to explain the nephroprotective impact of DBZ in the kidneys through decrease inflammation, oxidative stress, and apoptotic pathway. The current investigation found that renal IRI raised tissue levels of KIM1, IL-1β, TNF-α, F2-isoprostane, and caspase-3 with decreased the level of GSH in kidneys of rats. Interestingly, pre-treated with DBZ reduced KIM1levels, inflammatory markers (IL-1β, TNF-α), oxidative stress marker (F2-isoprostane) and apoptotic marker (Caspase-3) with increased antioxidant (GSH). The study by [27] revealed that the increase the level KIM-1 in the proximal tubule in early damage of kidney have advantage as a sensitive, specific and ideal biomarker marker for early diagnosis of kidney injury including acute kidney injury (AKI) and as a predictor of prognosis. The findings suggest that Dibenzazepin has a protective impact on kidney function parameters (KIM) following renal IRI. The important parameters for detection of inflammation in RIRI are (IL-1β and TNF-α) [16] demonstrate that the elevation of the inflammatory cytokines (IL-1β and TNF-α) as a result of hypoxia due to decreased in renal supply which cause infiltrate of different inflammatory cells into the injured tissue. The decrease in the levels of inflammatory factors explained by [28-17] said that cisplatin causes nephrotoxicity in rats by activating NFκB, which leads to increased production of proinflammatory cytokines such IL-1β and TNFα. Pre-treatment with dibenzazepine reduces nuclear translocation of NF-κB and significantly suppresses production of inflammatory markers TNFα and IL-1β. The imbalance in antioxidant agents and the amount of ROS production causes the accumulation of ROS, which may cause cellular damage. This process is known as oxidative stress and plays a key role in various cellular signalling pathways by destroying lipids, proteins, and DNA, resulting in cell death [29]. During ischemia, there is a significant reduction in oxygen supply, which causes a decrease cellular ATP synthesis and lactate build-up, resulting in acidosis. As a result, Na+/K+ATPases, Na+/H+, and Ca2+-ATPase pumps become dysfunctional, leading in the build-up of sodium, hydrogen, and calcium in the cytoplasm and consequent hyper-osmolality, an increase in water transport across

cell membranes, and cellular swelling. Furthermore, the overproduction of ROS during ischemia/reperfusion through production of several enzymes able to decrease molecular oxygen producing all of which contribute to oxidative stress, which plays a significant role in organ damage following I/R by triggering apoptosis and lipid peroxidation [30]. The nephroprotective effects of pre-treated DBZ in cisplatin-induced kidney damage in rats was achieved via significantly lowered oxidative stress, inflammation, and apoptotic indicators, as well as the Notch signalling pathway these findings reflect that the Notch pathway can play important role in the pathogenesis of cisplatin nephrotoxicity [28]. Another study by [31] have demonstrated that during IRI, the inflammation and tubular cell injury can trigger by burst of (ROS) ,therefore, reducing oxidative stress, can prevent damage in IRI by assessed F2-isoprostane. The current study showed significant decreased in Glutathione (GSH) in renal tissue of the control group and vehicle group in comparison with the sham group after renal IRI, this result agreement with [32] which demonstrated that GSH levels significantly reduced in RIRI and vehicle groups when compared with the sham group in response to renal ischemia reperfusion, suggesting that this results due to deterioration in the ability of kidney to function as an antioxidant. The [33] shown that when renal IRI occurs, the mitochondria of kidney cells are damaged lead to reduce the activities of GSH and SOD lead to unable to remove ROS and aggravating kidney damage. The pre-treated group with DBZ significantly increased the level of GSH and that consist with research by [34-28] which shown that the group which pre-treatment with DBZ in rats induced nephrotoxicity by cisplatin-injected cause improved the oxidative stress by significantly raising catalase and GSH. The significant increase of Caspase-3 in current study is in agreement with [35] shown that after thirty min of renal ischemia after that two hours of reperfusion in rats model the significant increase in Caspase-3level with significant decrease in Bcl-2 which explain the results that apoptosis is a conclusive process in renal IRI and the stimulation of caspase-3 have important role for programmed cell death because the equilibrium between antiapoptotic (Bcl-2) proteins and pro-apoptotic (Bax) is essential for the survival of cells, the disruption of this equilibrium in IRI through down regulating Bcl-2 levels and up regulating Bax expression, promoting apoptosis. The significant decreased in Caspase-3level in DBZ pre-treated group shown the nephroprotective effect of DBZ after renal IRI. Furthermore, histological examinations confirmed DBZ's kidney-protective properties. These examination showed a substantial reduction in renal tissue damage, including decrease in damage of renal tubules, infiltration of inflammatory cells, in rats treated with DBZ compared to control and vehicle groups. Previous study by [34] have reported this conclusion which said that pre-treatment of cisplatin-injected rats with DBZ significantly reduced cisplatin-induced alterations in nephrotoxicity indicators and the histological architecture of the kidney, another research by [36] revealed that the Notch inhibitor DBZ can ameliorate the expression of fibrotic markers and severity of renal Fibrosis caused by unilateral ureter blockage in mice. Fibrosis caused by unilateral ureter blockage in mice. Moreover, the Notch regulated gene overexpression is responsible for fibrosis of kidney and disease progression, the dysfunctional mitochondria leading to cell death and dedifferentiation [37]. The analysis of results revealed that the Dibenzazepin pre-treated group showed significantly lower level of Notch gene expression in renal tissue level and this result is in agreement with the previous study by [38] which demonstrated that ischemic mice were treated with the γ-secretase inhibitor DBZ, which inhibited Notch signalling and particularly decreased expression of Notch 1 ligands Jagged-1. Duan and Qin said that the blocking of Notch signalling pathway by using γ-secretase inhibitors decreases the amounts of Notch signalling components in podocytes and nephrons [39].

CONCLUSIONS

DBZ shown a nephroprotective effect due to ischemia/reperfusion (I/R). DBZ (γ-secretase inhibitor) exhibited anti-apoptotic, antioxidant, and anti-inflammatory characteristics by significant decreased in inflammatory, oxidative apoptotic markers and Notch1 and Jagged-1 expression in kidney tissues after renal ischemia/reperfusion damage. Therefore it may be a promising treatment people with RIRI.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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