ORIGINAL ARTICLE

CONTENTS

Silibinin nephroprotective effects in renal ischemia reperfusion injury in rats *via* regulation of Notch-1

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ABSTRACT

Aim: To evaluate nephroprotective effects of Silibinin in renal ischemia -reperfusion injury in rats by regulation of Notch-1 and Jagged-1.

Materials and Methods: 28 male Wistar albino rats were randomly divided into four groups, (seven rats / group): sham group (laparotomy only), control group (ischemia for 30 min / 2 hrs reperfusion), DMSO vehicle group (rats were injected intraperitoneally with 10% DMSO 1 hour before ischemia, then ischemia for 30 min / 2 hrs reperfusion, Silibinin treatment group (rats were injected with Silibinin (60 mg/kg) intraperitoneally 1 hour before ischemia, then "ischemia for 30 min / 2 hrs reperfusion. The kidneys were harvested after 2 hours of reperfusion for assessment of kidney injury molecule-1 (KIM-1), interleukin 1 β (IL-1β), TNF-α, F2-Isoprostane, and glutathione (GSH) by ELISA technique; Notch-1 and Jagged-1 mRNA levels were assessed by reverse transcription-polymerase chain reaction (RT-PCR), BAX and Bcl2 protein expression were assessed by immunohistochemistry (IHC).

Results: Silibinin demonstrated considerable amelioration of renal ischemia reperfusion injury as evidenced by the significant reduction of KIM-1, IL-1β, TNF α, F2-Isoprostane, in rat kidneys pretreated with Silibinin, GSH level was significantly elevated in rat kidneys pretreated with silibinin. Furthermore silibinin pretreatment significantly reduced Notch-1 and Jagged-1 mRNA expression after renal ischemia reperfusion.

Conclusions: Silibinin offers protection against renal ischemia reperfusion injury due to its antioxidant, anti-inflammatory, and anti-apoptotic effects, those effects are associated with the downregulation of Notch-1 and its ligand, Jagged-1.

KEY WORDS: renal ischemia reperfusion injury, silibinin, Notch-1, Jagged-1

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ABBREVIATIONS

RIRI: Renal Ischemia Reperfusion Injury
AKI: Acute Kidney Injury
DGF: Delayed Graft Function
ROS: Reactive Oxygen Species
MAM: Mastermind Transcription Facto
NICD: Notch Intracellular Domain
Hes: hairy and enhancer of split
Hey: hairy/enhancer-of-split linked with YRPW motif
NF-κB: nuclear factor kappa B
ELISA: Enzyme Linked Immunosorbent Assay

INTRODUCTION

Acute kidney injury (AKI) is characterized by a sudden decrease in kidney function, which leads to internal milieu disruptions and uremic toxin retention, which primarily affects the immunological, cardiovascular, and neurological systems [1]. One of the major contributors to acute kidney injury (AKI) is renal ischemia-reperfusion injury (RIRI). It often occurs during kidney transplantation, where it may cause delayed graft function (DGF) that can lead to loss of vital kidney tissue and activates immune responses that can provoke rejection, ultimately leading to the loss of the graft [2]. In addition to kidney transplants, RIRI can also develop in other conditions associated with reduced blood flow or oxygen supply, such as thrombotic disorders, cardiac surgeries, traumas, and sepsis [3-4]. Reperfusion of an ischemic kidney worsens oxidative stress and inflammation, resulting in damage to DNA and proteins, ultimately causing cell apoptosis and necrosis. The main mechanisms behind this process include the release of pro-inflammatory substances, activation of genes related to apoptosis, production of reactive oxygen species (ROS), and excess calcium levels [5-6]**.** The Notch signaling pathway plays a crucial role in regulating important developmental processes, such as lateral inhibition, lineage decisions, and the formation of boundaries, in addition to contributing to homeostasis and regeneration in adult tissues. It is a type of juxtacrine signaling pathway where both the receptors and ligands are transmembrane proteins that facilitate communication between neighboring

cells. For trans-signaling events to occur, direct contact between the cells is necessary, allowing the receptor to bind with its ligand [7]. Signal transduction occurs at the moment of ligand binding to the receptor which is followed by two successive proteolytic cleavages leading to the release of the Notch Intracellular Domain (NICD) which Translocates to the nucleus to enhance transcription of Notch target genes [8]. While Notch signaling is notably reduced in the adult kidney, research indicates that both acute and chronic kidney injuries can lead to a reactivation of Notch signaling [9] analysis of the kidneys of mice after induction of renal IRI showed an increased expression of processed Notch-2, Delta-1, and Hes-1 mRNA and protein. This indicates that Notch2 is involved in the proliferation of epithelial cells [10]. Silibinin is the primary biologically active component of silymarin which is a specific combination of polyphenolic flavonoids extracted from milk thistle extract. It is a mixture of two diastereomers (A and B). Silibinin's potent anti-hepatotoxic effects have led to its widespread use as a dietary supplement due to its great human acceptability. Milk thistle plant has been utilized for liver disease therapy for over 2,000 years [11]. The nephroprotective effects of silibinin in cisplatin induced AKI were examined by study which revealed that silibinin ameliorated cisplatin-induced AKI via decreasing ROS-mediated MAPK signaling pathway activation, which was confirmed using the inhibitor N-acetylcysteine. Moreover, the protective effect of silibinin against cisplatin-induced ROS generation through the antioxidant transcription factor nuclear factor-erythroid 2-related factor 1 (Nfe2l1), rather than Nfe2l2, mediates HO1 expression [12]. Limited number of studies were performed to examine the nephroprotective effects of silibinin in renal IRI. Deng et al have shown that silibinin ameliorated renal dysfunction and inflammation in mice model of renal IRI by inhibiting ferroptosis through binding to ferritin heavy chain 1 (FTH1) [13].

AIM

The present study aims to investigate the potential nephroprotective effects of silibinin in renal ischemia reperfusion injury rat model and to investigate the role of Notch signaling pathway in mediating these effects.

MATERIALS AD METHODS

ANIMAL PREPARATION AND ETHICAL CONSIDERATIONS

The research received approval from the Institutional Animal Care and Use Committee (IACUC) at Kufa University. A total of twenty-eight male Wistar albino rats, each weighing between 250-300 grams, were obtained from the Animal Resources Centre at the College of Science at Kufa University. The rats were housed in the university's animal facility, maintained at a controlled environment of 25±2°C and 60-65% humidity, with a 12-hour light/dark cycle. They were provided with unrestricted access to water and standard laboratory chow.

STUDY DESIGN

Following a two-week acclimation period, the rats were divided into four groups, each comprising seven rats as follows: Sham group: rats were subjected to anesthesia and surgery without the induction of renal ischemia, control group: rats were subjected to 30 minutes of bilateral renal pedicle clamping to induce renal ischemia, followed by a two-hour reperfusion period, DMSO vehicle group in which rats were injected intraperitoneally (i.p.) with 10% DMSO the vehicle for silibinin 1 hour before induction of ischemia followed by bilateral renal pedicles clumping for 30 minutes and then reperfusion for two hours, and silibinin treatment group: rats were injected with silibinin (60 mg/kg) [14] dissolved in 10% DMSO i.p. 1 hour before induction of ischemia followed by bilateral renal pedicles clumping for 30 minutes and then reperfusion for two hours.

BILATERAL RENAL ISCHEMIA REPERFUSION INJURY MODEL

Rats were anesthetized using an intraperitoneal injection of a mixed solution containing ketamine at a dosage of 100 mg/kg and xylazine at 10 mg/kg. They were then placed in cages maintained at a stable temperature to prevent hypothermia. Once the loss of righting reflex was confirmed, the rats were laid on their backs, and their limbs and tail were secured with stickers to ensure stability during the surgical procedure. After making a midline laparotomy incision, the intestines were retracted to expose the abdominal cavity and renal pedicles [15]. The renal pedicles were carefully separated, and microvascular clamps were applied around them. Within one to two minutes, the kidney's color shifted from red to deep purple, indicating ischemia [15]. After a thirty-minute period, the clamps were taken off to allow blood flow to return, initiating the reperfusion phase. The abdominal incision was then closed using 3/0 silk sutures. Following two hours of reperfusion, the rats were anesthetized once more, and both kidneys were excised. The rats were then euthanized using an excessive dose of ketamine. The left kidney was kept in liquid nitrogen for the assessment of experimental parameters.

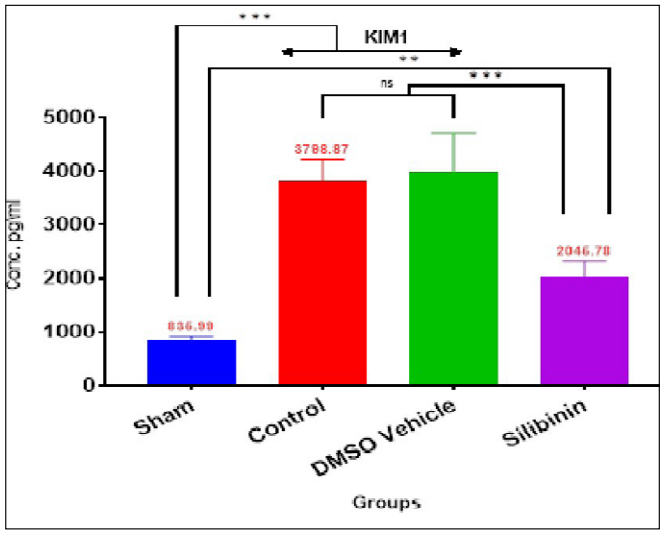

Fig. 1. Effect of silibinin on KIM-1 renal tissue concentration (pg/ml), no. of animals in each group=7
**p<0.01, ***p< 0.001
Source: Own materials

EVALUATION OF KIM-1, IL-1B, TNF A, F2 ISOPROSTANE, AND GSH RENAL TISSUE CONCENTRATION BY ELISA TECHNIQUE

After surgically removing the left kidney from each rat, the organ was rinsed with cold normal saline to ensure that all blood clots were eliminated. Next, the kidney was cut into two halves. One half was subjected to homogenization using a high-intensity ultrasonic liquid processor, mixed in a 1:10 (w/v) dilution of phosphate-buffered saline (PBS) that included 1% Triton X-100 and a cocktail of protease inhibitors. The homogenate was subsequently centrifuged at 4°C for 15 minutes at 22,132 × g. The supernatant was collected and distributed into Eppendorf tubes for further analysis of KIM1, IL-1β, and TNF-α, GSH, and F2 Isoprostane using the ELISA technique. The kits were purchased from SunLong Biotech Co.LTD, China. (KIM-1 kit Catalogue No.: SL0433Ra, TNF-α kit Catalog No.: HS-EL0012Ra, IL-1β kit Catalog No.: EL0040Ra, F2 isoprostane kit catalogue NO.:SLD2059Ra, GSH kit Catalogue No.:SL1410Ra).

IMMUNOHISTOCHEMISTRY (IHC) ANALYSIS OF BAX AND BCL2

The Immunohistochemistry analysis was performed in The Middle Euphrates Cancer Research Unit at Kufa University. Formalin fixed paraffin embedded section slides were used for this method. Primary antibodies were used to prob the sections against the required antigens. The primary antibodies were purchased from Elabscience®, USA (BAX catalog NO. E-AB-13814), (Bcl2 catalog NO. E-AB-22004).

Labeled secondary antibodies were then added to assess the target protein expression. An enzymatic label was used to detect the antigen-antibody combination. The IHC analysis depended on labelled streptavidin-biotin staining method (LSAB). H score calculations depends on the intensity of staining and the proportion of cells affected; the staining intensity is given a score range from (0-3). Zero for no stain, 1 denotes weak intensity staining, 2 denotes moderate staining, while 3 represent strong staining, the H score for each sample is obtained by multiplying the staining intensity score by the proportion of cells affected.

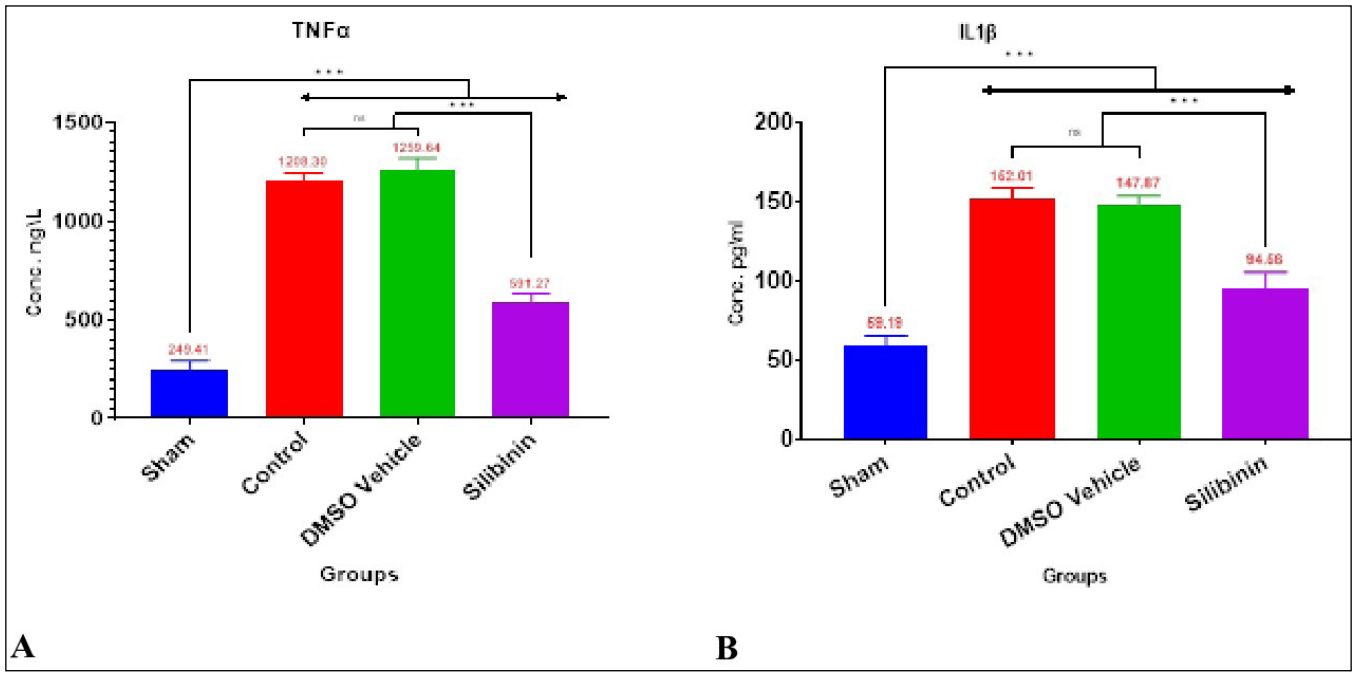

Fig. 2. Effect of silibinin on (A): TNFα renal tissue concentration (pg/ml), (B): IL1β renal tissue concentration, no. of animals in each group=7, **p<0.01, ***p<0.001
Source: Own materials

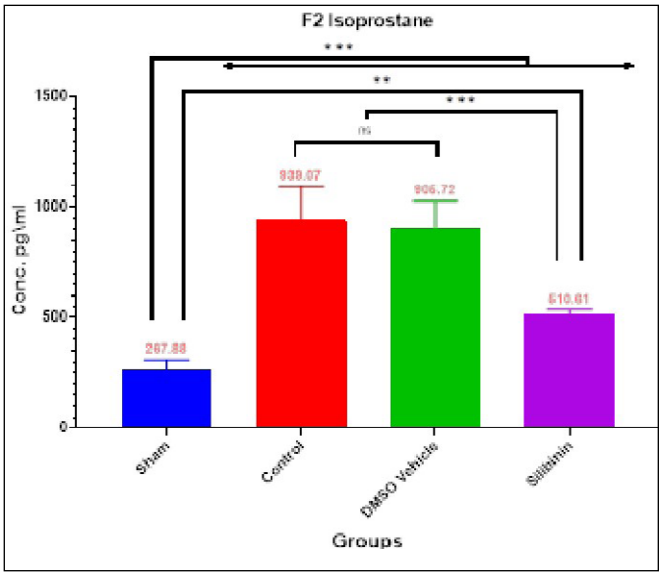

Fig 3. Effect of silibinin on F2 Isoprostane renal tissue concentration (pg/ml), no. of animals in each group=7
**p<0.01, ***p<0.001
Source: Own materials

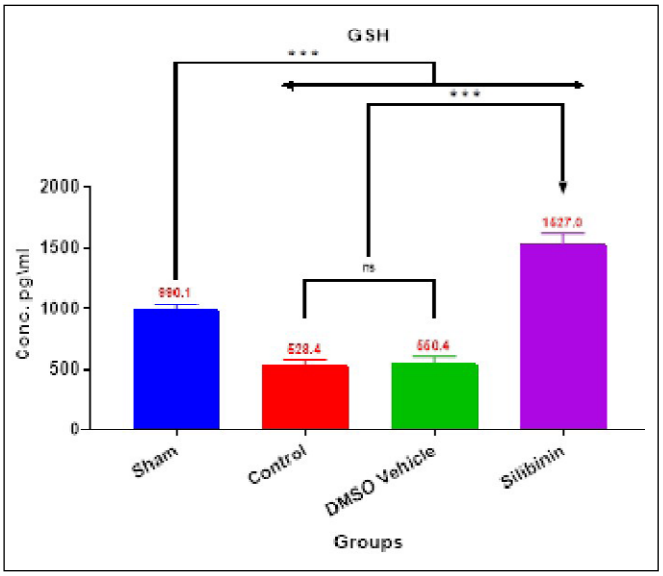

Fig. 4. Effect of silibinin on GSH renal tissue concentration (pg/ml), no. of animals in each group=7
**p<0.01, ***p< 0.001
Source: Own materials

ESTIMATION OF NOTCH1 AND JAGGED1 MRNA EXPRESSION BY RT-PCR

We used the quantitative real-time PCR (qRT-PCR) technique to accurately evaluate the expression levels of Notch1 and Jagged1 mRNA. Initially, we extracted total RNA with precision using the Easy-spin™ (DNA-free) total RNA extraction kit. The high-quality RNA was subsequently converted into complementary DNA (cDNA) with the AddScript cDNA Synthesis Kit. For the quantitative real-time PCR process, we employed the GoTaq® RT-qPCR System to guarantee dependable results and specific primers: Notch1 for: CACCCATGACCACTACCCAGTT, Notch1 rev: CCTCGGACCAATCAGAGATGTT, Jagged1 for: AACTGGTACCGGTGCGAA, Jagged 1 rev: TGATGCAAGATCTCCCTGAAAC [16] and GAPDH for: ATGACTCTACCCACGGCAAG, GAPDH rev: CTGGAAGATGGTGATGGGTT [17].

STATISTICAL ANALYSIS

Statistical analysis of this study was performed by Graph Pad Prism version 8 software. Parametric variables were tested using the one-way ANOVA test with multiple comparisons between groups being made by Post Hoc. tukey Test.

RESULTS

SILIBININ ATTENUATES KIM-1 RAT RENAL TISSUE CONCENTRATION AFTER RENAL I/R

KIM-1 renal tissue concentration was considerably ($p < 0.001$) increased after renal ischemia /reperfusion (renal I/R) in comparison with sham group. Notably, this increase was attenuated by silibinin pretreatment in a dose of (60 mg/kg) i.p., however, silibinin treatment group showed significant difference ($p < 0.001$) when compared to sham group (Fig. 1).

SILIBININ ATTENUATES IL-1B AND TNFA RENAL TISSUE CONCENTRATIONS AFTER RENAL I/R

Following renal ischemia/reperfusion, the concentration of both IL-1β and TNFα in the renal tissue of rats were significantly increased ($p < 0.001$). Pretreatment with silibinin at a dosage of 60 mg/kg administered i.p. notably reduced the levels of these markers. However, the silibinin treatment group still showed significantly elevated levels ($p < 0.001$) when compared to the sham group (Fig. 2A-B).

SILIBININ REDUCES F2 ISOPROSTANE RENAL TISSUE CONCENTRATION AFTER RENAL I/R

As shown in figure 4, sham group showed significantly lower F2 Isoprostane renal tissue concentration as compared to control group. However, silibinin treatment group was considerably lower $p<0.001$ than control group, but it was still significantly higher $p<0.001$ than sham group (Fig. 3).

SILIBININ IMPROVES GSH LEVELS IN RATS RENAL TISSUE AFTER RENAL I/R

Interestingly, I/R significantly ($p< 0.001$) decreased GSH rat renal tissue concentration relative to sham group. Pretreatment with silibinin in a dose of 60 mg/kg i.p. caused considerable ($p< 0.001$) elevation in GSH renal tissue concentration in comparison with control group, (Fig. 4).

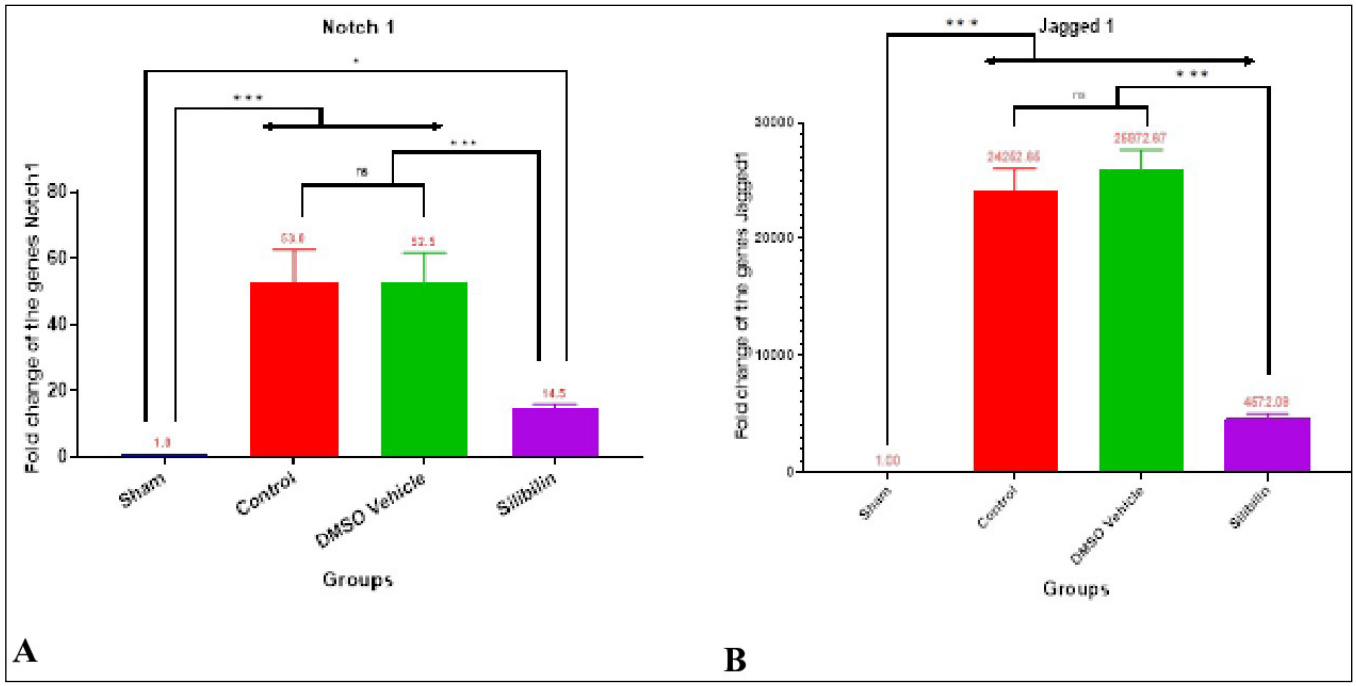

Fig. 5. Effect of silibinin on (A): Notch1 mRNA expression level in renal tissue, (B): Jagged-1 mRNA expression level in renal tissue, no. of animals in each group=7
**p<0.01, ***p<0.001
Source: Own materials

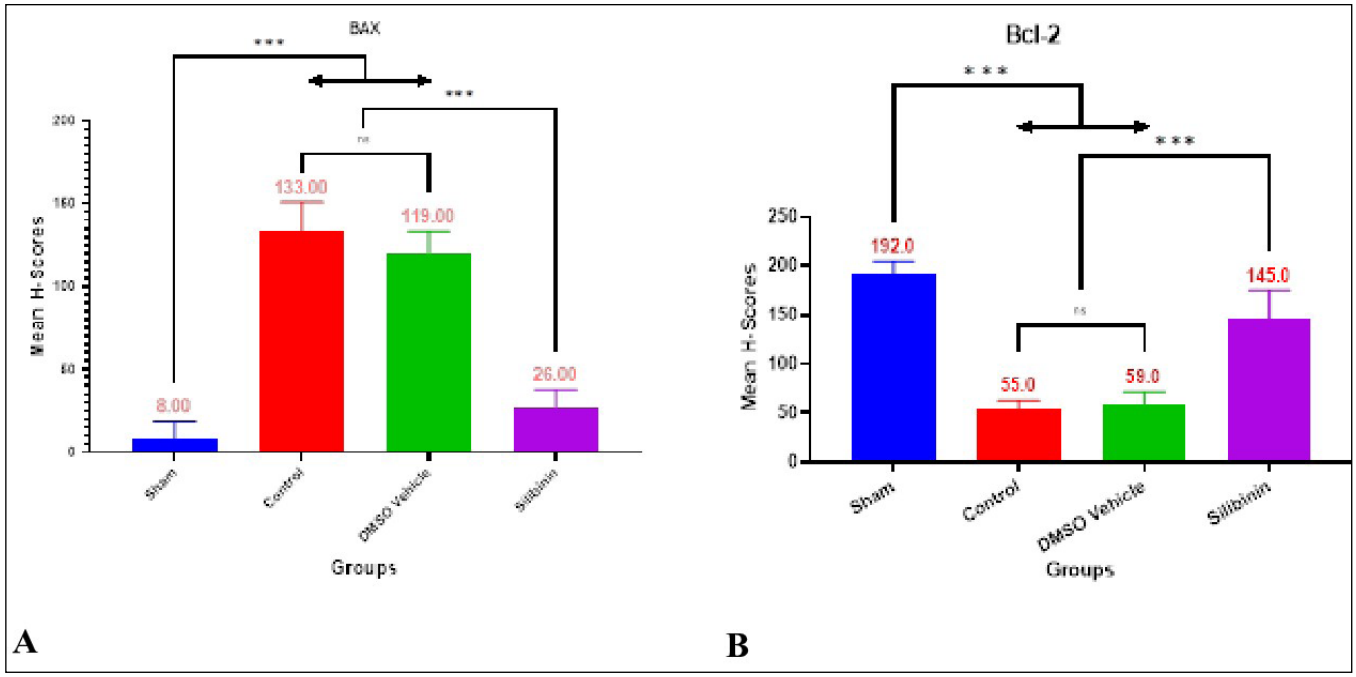

Fig. 6. Effect of silibinin on protein expression of (A): BAX, (B): Bcl2 in renal tissue, no. of animals in each group=7
**p<0.01, ***p<0.001
Source: Own materials

SILIBININ REDUCES MRNA EXPRESSION OF NOTCH-1 AND JAGGED-1 IN RATS RENAL TISSUE AFTER RENAL I/R

Both Notch-1 and its ligand Jagged-1 showed a significant increase p<0.001 in the renal tissue of rats following ischemia/reperfusion (I/R). This increase was notably reduced p<0.001 by pretreatment with silibinin in a dose of 60 mg/kg administered intraperitoneally (Fig. 5A-B).

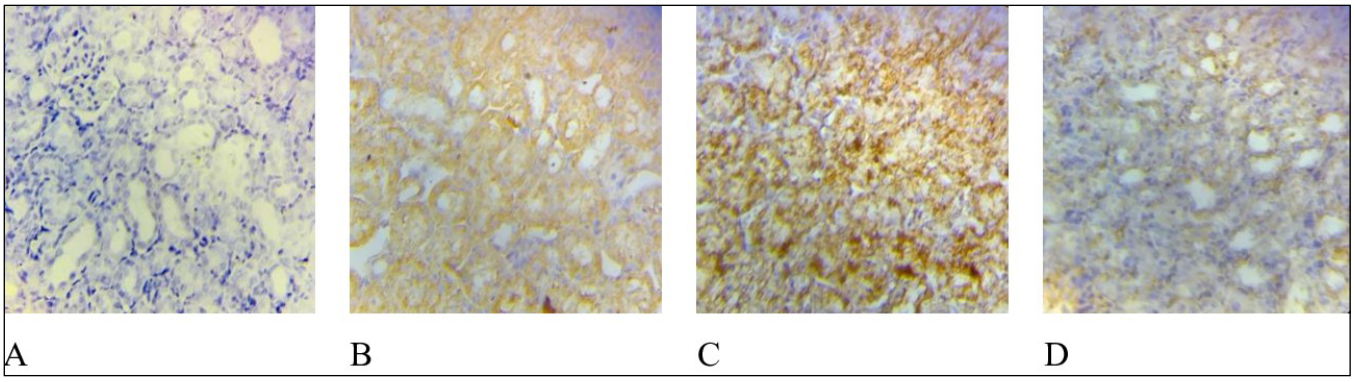

Fig. 7. BAX immunohistochemical staining, X400, (A): sham group, negative stain IHC, (B): Control group. Positive moderate intensity cytoplasmic staining in 80% of the examined cells, (C): DMSO Vehicle group. Positive moderate intensity cytoplasmic staining in 70% of the examined cells, (D): silibinin treatment group. Positive weak intensity cytoplasmic staining in 10% of the examined cells.
Source: Own materials

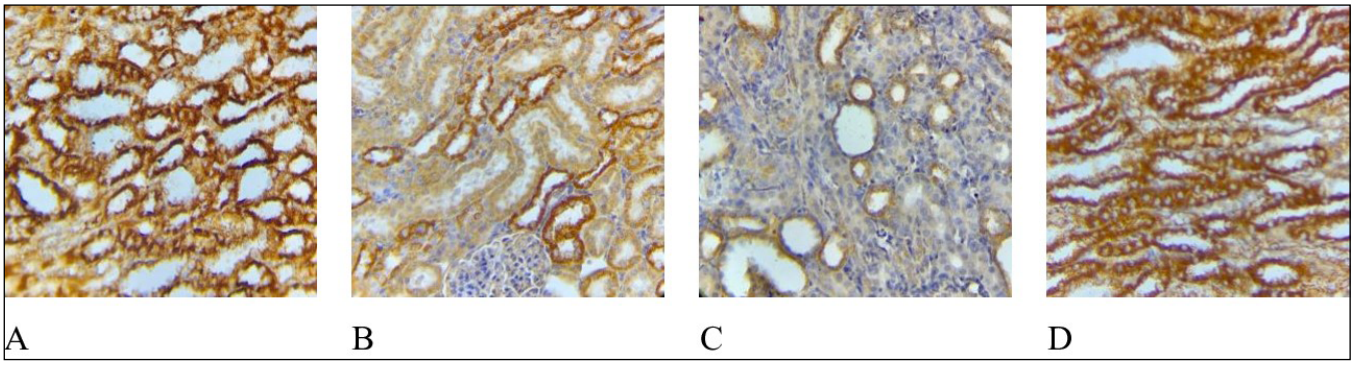

Fig. 8. Bcl2 immunohistochemical staining, X400, A: sham group Positive strong intensity cytoplasmic staining in 70% of the examined cells, B: control group. Positive strong intensity cytoplasmic staining in 35%% of the examined cells, C: DMSO vehicle group Positive moderate intensity cytoplasmic staining in 20% of the examined cells, D: silibinin treatment group, Positive strong intensity cytoplasmic staining in 60% of the examined cells.
Source: Own materials

SILIBININ ATTENUATES APOPTOSIS BY DECREASING BAX AND INCREASING BCL2 PROTEIN EXPRESSION

The expression level of apoptotic marker BAX in renal tissue of sham group was significantly ($p<0.001$) low in comparison with control group, while silibinin pretreatment caused considerable lowering ($p < 0.001$) in BAX protein expression when compared to control group (Fig. 6A, Fig. 7A-D). On the other hand, the anti-apoptotic marker Bcl2 protein expression is significantly decreased in control group in comparison with sham group, meanwhile, silibinin pretreatment showed significant $p<0.001$ elevation in Bcl2 protein expression in comparison with control group (Fig. 6B, Fig. 8A-D).

DISCUSSION

Earlier research has shown that renal ischemia-reperfusion injury (IRI) is strongly linked to oxidative stress and inflammatory reactions which are particularly related to the damage of renal tubular epithelial cells (TECs) that plays a significant role in the overall damage to the kidneys [18]**.** Numerous molecular pathways have been proposed to be involved in the pathological processes that result in cellular damage related to ischemia-reperfusion injury (IRI). Furthermore, basic research has identified various targets that may help slow the progression of kidney disease. Despite these promising discoveries, only a few have been effectively validated in clinical trials, and they do not provide a substantial alternative to dialysis or transplantation. In the current study, we evaluated the nephroprotective effects of silibinin in renal IRI and the association of Notch signalling pathway in mediating these effects. The present research indicated a notable increase in the levels of KIM-1 within renal tissue following renal ischemia/reperfusion (I/R). Since KIM-1 is absent in healthy kidney tissue and is uniquely produced in injured proximal tubules, it acts as a highly sensitive, specific, and accurate early marker of renal tubular damage [19], validating the efficacy of the IRI model used in this study, silibinin treatment in a dose of (60mg /kg) i.p.1 hour before induction of ischemia resulted in significant reduction in KIM-1 renal tissue level when compared

to control group and each of the vehicle groups which may reflects the nephroprotective effects of silibinin. This study comes into agreement with another study of renal IRI in mice, in which silymarin that is a mixture of both silibinin isomers was found to decrease KIM-1 protein expression in addition to other markers of oxidative stress, inflammation through inhibition of apoptotic pathways and NF-κB [20]**.** In this experimental study, comparisons between the sham and control groups showed that both TNF-α and IL1β levels were significantly increased in the control groups. This supports the notion that renal inflammation was present due to renal IRI. It has become clear that a substantial amount of the harm observed following renal ischemia-reperfusion (IR) is caused by oxidative stress in the mitochondria and the generation of reactive oxygen species (ROS) during the reperfusion phase. These ROS, such as superoxide, peroxynitrite, and hydrogen peroxide (H2O2), activate inflammatory cells, leading to the release of interleukins (ILs), tumor necrosis factor (TNF-α), and a variety of other inflammatory substances [21]. This elevation in inflammatory markers was significantly attenuated by silibinin pretreatment. This reduction in TNF-α level and other inflammatory cytokines could be due to the inhibition of NF-κB pathways which has been documented by many studies [20-22]. This study also assessed a marker of oxidative stress known as F2-isoprostane, which is produced through a non-enzymatic reaction resulting from the significant peroxidation of arachidonic acid due to free radicals. Research conducted by the National Institutes of Health (NIH) in the United States has recognized Isoprostane as reliable biomarkers for oxidative stress. Furthermore, the study illustrated that measuring bioactive F2-isoprostanes in bodily fluids provides a novel noninvasive method for investigating the biological effects of free radicals [23]. In the present study renal tissue concentration of F2-isoprostane was considerably elevated after renal I/R. meanwhile silibinin pretreatment significantly reduced F2 Isoprostane renal tissue concentration after renal IR which reflects the potential antioxidant capacity of silibinin. The antioxidant effects of silibinin were proposed by many studies. In cisplatin induced AKI model, silibinin decreased ROS-mediated MAPK signaling pathway activation, in addition silibinin countered the inhibition of nuclear factor-erythroid 2-related factor 1 (Nfe2l1) caused by cisplatin by influencing its transcription and modifications after translation [24]. Another marker of oxidative stress was evaluated in the current study which is glutathione (GSH). It inhibits lipid peroxidation and scavenges free radicals (LPO). Some detoxifying enzymes and antioxidants require GSH as a cofactor, it aids in the renewal of ascorbate and tocopherols [25]. Our study has shown a considerable reduction in GSH renal tissue concentration after renal IR which could be attributed to the depletion of the tissue antioxidative capacity by the huge amounts of ROS produced during the reperfusion phase as a result of the defect in the mitochondrial electron transport chain [26]. Interestingly, silibinin pretreatment significantly ameliorated GSH renal tissue concentration after renal IR. Our study agrees with another study that evaluated the nephroprotective effects of silibinin in renal IRI in mice showing significant elevation in GSH level in rat kidney tissue after IR in silibinin treated mice and in mouse renal tubule tissue culture treated with silibinin in addition to improving other markers of oxidative stress like MDA and SOD, those effects were suggested to be mediated through inhibiting ferroptosis by binding Ferritin heavy chain 1 (FTH1) [27]. Previous research has indicated a close relationship between apoptosis and the pathological process of renal ischemia-reperfusion injury (IRI) [28]. The fate of cells following renal IRI is determined by the balance between the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2, both of which are part of the Bcl-2 family [29]. Excessive reactive oxygen species (ROS) trigger stress pathways in both the mitochondria and the endoplasmic reticulum. When mitochondrial damage occurs, the rise in Bax levels and the fall in Bcl-2 levels alter the Bax to Bcl-2 ratio in the outer mitochondrial membrane, leading to the release of cytochrome c. This release activates the apoptosis executioner enzyme caspase-3, ultimately resulting in mitochondrial-dependent apoptosis [30]**.** In this experimental study, immunohistochemical analysis of the apoptotic activator BAX and its antagonist Bcl2 revealed significant upregulation in BAX renal tissue protein expression level after renal IR while Bcl2 was significantly downregulated after renal IR. The results of this study clearly align with a previous study on the protective effects of Allicin in rat model of renal ischemia reperfusion injury which documented the increase in caspase-3, BAX and the decrease in Bcl2 renal tissue protein expression after renal IR [31]. On the other hand, silibinin pre-treatment resulted in significant increase in Bcl2 renal tissue protein expression while decreasing BAX protein expression in renal tissue after IR. Silibinin may improve apoptosis by decreasing oxidative stress and scavenging ROS, this can prevent the activation of mitochondrial apoptotic pathways by reducing BAX and enhancing Bcl2 levels [32]. The findings of this study agree with another study which assessed the nephroprotective effects of silymarin in renal ischemia reperfusion injury in mice which have shown that silymarin enhanced Bcl2 protein expression while lowered both

Bax and cleaved caspase-3 [33]. In order to assess the effect of silibinin on Notch-1 signaling pathway, we estimated Notch-1 and its ligand Jagged-1 m RNA expression in all study groups. Our results have shown significant elevation in Notch-1 and Jagged-1 m RNA expression after IR. This study comes in line with a previous study which evaluated the nephroprotective effects of Notch inhibitor DAPT in renal I/R injury induced in diabetic rats showing that both Notch-1 and its ligand Jagged-1 m RNA expression in renal tissues were elevated after renal I/R [34]. Notably, pretreatment with silibinin resulted in a significant reduction of Notch-1 and Jagged-1 mRNA levels in renal tissues following renal IR. In an *in vivo* and *in vitro* model of hepatocellular carcinoma, silibinin exerted potent antitumor activity which was accompanied by Notch-1 down regulation [35]**.** The inhibition of oxidative stress and NF-κB activation by silibinin [36] could provide a reasonable explanation to the down regulation of Notch-1 by this supplement since they are documented regulators of Notch-1 and Jagged-1 [37].

CONCLUSIONS

Results of this study may provide additional evidence regarding the role of inflammation, oxidative stress, and apoptosis in the development of renal ischemia/reperfusion (I/R) injury, indicating that the Notch-1 signaling pathway may be involved in these processes. Silibinin appears to offer protective benefits against renal I/R injury due to its antioxidant, anti-inflammatory, and anti-apoptotic properties. Furthermore, the protective effects of silibinin were linked to a decrease in the expression of Notch-1 and its ligand Jagged-1.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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