

Effects of Vinpocetine on Renal Ischemia Reperfusion Injury in a Male Rat Model

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ABSTRACT

Background: Ischemia-reperfusion injury (IRI) occurs when the blood supply to tissue diminishes and subsequently restore. The basic pathophysiology involves reactive oxygen species, robust inflammation, and induction of cell death pathways terminated in organ dysfunction. Renal ischemia-reperfusion is a major cause of acute kidney injury (AKI) that occurs in clinical conditions implicates the challenge such as in transplantation surgery, renal artery stenosis, sepsis, cardiopulmonary bypass, and trauma. Objectives: This study was undertaken to investigate the potential protective effect of Vinpocetine (alkaloid extract of a periwinkle plant) on bilateral renal IRI in male rats, through study the effectiveness of Vinpocetine on renal function, it's possible role in modulation of the inflammatory cascade, and its anti-oxidant & anti apoptotic effect. Methods: A twenty-adult male Wistar albino rats, weights about (220-260 gm), aged 8-12 weeks, after two weeks of acclimatization, the rats were randomly divided into four groups (5 rats in each group) as follows: Sham group: rats underwent the same anesthetic and surgical procedure except clamping of the bilateral renal artery. Control group: rats subjected to the bilateral renal ischemia for 30 min by clamping renal pedicles and reperfusion for 2 hours. Vehicle group: rats received 10 % DMSO by I.P route before 30 min of induction of bilateral renal ischemia for 30 min by clamping of the pedicles and then 2 hours reperfusion. Vinpocetine pretreated group: rats pretreated with vinpocetine 10 mg/kg I.P, 30 min before clamping of the renal pedicles and then underwent bilateral renal ischemia for 30 min and then reperfusion for 2 hours. At the end of the procedure, all rats were sacrificed and

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tissue and blood samples were collected. Samples subjected to various outcomes measurements including renal function test (serum urea and creatinine), renal level of inflammatory mediators (TNF α , IL-6), and total antioxidant capacity (TAC) were carried via the ELISA technique. Immunohistochemistry also was done to investigate proapoptotic protein (BAX) and antiapoptotic protein (BCL-2) and microscopical examinations were performed to determine the parenchyma injury. Results: The renal ischemia-reperfusion injury (RIRI) caused significant ($p \leq 0.05$) higher serum level of Urea & Creatinine and renal level of TNF α , IL-6, and BAX in control (+IR) and vehicle groups than those in the sham group. On the other hand, the renal level of TAC and Bcl-2 in both control and vehicle groups are significantly ($p \leq 0.05$) lower than those in the sham group. While pretreated vinpo group showed significantly ($p \leq 0.05$) lower serum level of urea S. Cr and lower renal level of TNF α , IL-6, and BAX in comparison to those in control and vehicle groups, besides the study, showed that the level of renal TAC and Bcl-2 in vinpo pretreated group significantly ($p \leq 0.05$) higher than that in control and vehicle groups. Histopathological findings of renal tissue in the vinpo pretreated group demonstrated significantly ($p \leq 0.01$) lower grade of architecture abnormality and severity of tubular damage when compared with control and vehicle groups. Conclusions: The present study found that Vinpocetine pretreatment attenuated renal IR injury in a rat model, mainly via improving renal function, oxidative modulating effects, suppressing inflammation, and decreasing cell apoptosis.

INTRODUCTION

Ischemia-reperfusion injury (IRI) results when blood flow restoring to the tissue that has been previously suffered from diminishing blood supply [1]. This restoration of blood paradoxically exacerbated the injury due to higher production in reactive oxygen species (ROS) and amplify several serious events concluding the oxidative stress, inflammation, and apoptosis [2]. IRI is one of the major causes of acute kidney injury (AKI) with a high mortality rate. It has an important contribution to the progression of chronic kidney disease and end-stage renal disease. Clinically, the wide condition in which kidneys suffering IRI, such as vascular and cardiac surgery, trauma, resuscitation circulatory arrest endovascular disease involving chronic renal artery stenosis, renal artery thrombosis, embolism, or atherosclerosis, and kidney transplantation [3–5]. Approximately 66% of AKI is induced by IRI or acute tubular necrosis. According to a recent meta-analysis which depending on the precise definition of kidney disease improving global outcome

(KDIGO) guidelines, 23% AKI incidence occurs during staying time in the hospital, while reports approximately 50–80% patients mortality with severe AKI [6]. Each year around 1.7 million people are believed to die from AKI [7]. Moreover, AKI is associated with short and long-term mortality, morbidity accompanied by costly treatment, and time of staying in hospital [8]. Hypoxia, glucose depletion, acidosis, and ROS production contribute to apoptosis and active necrosis [9]. The core of injury of AKI is sterile inflammation mediated by cytokines and leukocytes that are followed by tissue stress and necrosis [4,10]. The TNF- α secreted primarily by immune cells including macrophages, natural killer cells, and T-lymphocyte accordingly mediates regulation to immune responses, proinflammatory, and hematopoietic activity [11]. IL-6 is dominantly released from the kidney during the early time of reperfusion [12]. Several studies add a significant correlation between AKI and IL-6 therefore, may be considered beneficial to use as a biomarker or therapeutic target in ischemic AKI [13,14]. As when

blocking the trans-signaling of IL-6 is sufficient to decrease inflammatory reaction[14-15]. Apoptosis occurs in response to hypoxic stress which results from ischemia and production of ROS from reperfusion[16]. Hypoxic stress induces both the extrinsic and intrinsic pathways of apoptosis[5]. BCL2 AND BAX are apoptotic mediators and related to IRI [17]. Bax inhibitor act as the adapter for the apoptotic signal of mitochondria through preserving morphology and function of its, consequently keeping the function of renal tubule in AKI[18]. Vinpocetine (vinpo) is an alkaloid derived from the leaves of the *Vinca minor* periwinkle plant [19]. In 1978 in Hungary, Vinpocetine (Cavinton®) was firstly marketed and has been used worldwide in countries of Asia and Europe for treating memory disturbances, stroke, and senile dementia. Currently, different products containing Vinpocetine are used worldwide as dietary supplements [20]. To date, there are no reports of side effects, toxicity, or contraindications linked to the use of Vinpocetine at a therapeutic dose. So make it an interesting compound to explore therapeutic application[21]. Vinpocetine has several pharmacological activities. The major one is dilation of cerebral blood vessels and improving blood supply also boost the ATP production in neuron through increase cerebral uptake of glucose and oxygen [22-23]. Vinpocetine has different important mechanisms of action. The important mechanism of action is the anti-inflammatory effect through inhibition of IKK and subsequently inhibition NF- κ B activation and translocation. NF- κ B represents a major signaling pathway to gene expression and the production of proinflammatory cytokines [24]. Vinpo is considered as a phosphodiesterase type-1 (PDE1) inhibitor, which facilitates vasodilatation thereby elevating the level of vasodilators proteins cGMP and cAMP. Furthermore, it has a voltage-gated sodium channel blockade effect [24,25]. Also, it has antioxidant effects via decrease ROS, removal of free radicals, modulation of dopamine metabolism, and decrease vascular thrombosis [26-27]. Vinpocetine has been demonstrating an analgesic effect by decrease pain in different experimental models[28-30]. Also, Vinpocetine revealed antidepressant effect in an animal study [31]. In cardiac diseases Vinpocetine effectively suppressed cardiac hypertrophy and fibrosis [24]. While Vinpocetine has an antiapoptotic effect in the testicular IR rat model [27]. Vinpocetine protects various cells from ischemic injury[24]. Best to our knowledge there is no study was done to explore the reno-protective effect of Vinpocetine in bilateral renal IRI, accordingly the present study was done to highlight and demonstrate this effect.

MATERIALS AND METHODS

The site and ethical consideration of the research

The study was done in the department of pharmacology and therapeutic and Middle Euphrates Unit for Cancer Researches, Faculty of Medicine\University of Kufa. Animal care likewise handling, feeding, treatment, scarifications, and surgery operation criteria were performed according to the Institutional Animal Care and Use Committee (IACUC) in Kufa University.

Animal grouping

A Twenty adult male Wistar albino rats weighted about (220-260 gm), aged 8-12 weeks, and were brought from the Center of Control and Pharmaceutical research/Ministry of health. Animals' were harbored in the animal house of the Faculty of Science/ University of

Kufa with a temperature-controlled 20-25°C and 60-65% humidity with a fitted 12 hrs light and 12 hrs dark cycles for 14 days before the start of the procedures. Also, the rats were free to access food and water. In this study, the rats were divided randomly into 4 equal groups, 5 rats in each group, and like the following:

1. Sham group

All 5 rats underwent the same anesthetic and surgical procedures for an identical time for ischemia and reperfusion without ischemia-reperfusion induction. Renal tissues and blood samples were collected.

2. Control group

All 5 rats underwent median laparotomy under anesthesia, followed by 30min bilateral renal ischemia, then renal tissues and blood samples were collected after 2h of reperfusion[32,33].

3. Vehicle group

All 5 rats were pretreated with an intraperitoneal injection of DMSO 30 min before the IRI and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours [33].

4. Vinpocetine treated group

All 5 rats were pretreated by intraperitoneal injection of vinpocetine 10 mg/kg 30 min[34] before the IRI and undergo bilateral renal ischemia for 30 min and reperfusion for 2 hours.

Renal ischemia-reperfusion injury rat model

Rats were anesthetized with ketamine (in a dose of 100 mg/kg) and xylazine (10 mg /kg) via IP injection. Then after 5-10 min post-anesthesia, the animal was stabilized by limb sticking on back position, followed by abdominal shaving and disinfectant application. The reflex monitoring was done by tail and leg pinching to ensure anesthesia[35]. Then midline laparotomy was done and the intestine components were put out its anatomical place to facilitate the renal pedicle clamping. The ischemic insult was done according to (bilateral model of 30 min. ischemia then 2 hr. reperfusion). Ischemia started via clamping to hole pedicles of both kidneys, then abdominal covered by gauze soaked in normal saline, animals body temperatures were monitored and maintain it by using a hot blanket. After ending the desired ischemic time(30 min.),the clamps were removed and blood reperfusion remains for 2 hours with suturing of the abdomen. Fluid resuscitation was done with 1 ml of normal saline directly to the abdomen to prevent tissue dehydration and maintain hemodynamic status[36]. The left kidney was divided into 2 halves. The first half was kept in a deep freeze for assessment. While the second half was inserted in 10% formalin then embedded in paraffin for histopathological and immunohistochemical assessment.

Preparation of the drug

Vinpocetine (CAS NO 42971-09-5) was purchased from Med Chem Express, USA Company. Vinpocetine was prepared immediately before using by dissolved in DMSO according to manufacturer instructions.

Assessment of kidney function

The blood sample (2.5-4 ml) was collected from each rat by cardiac puncture and put directly into plain tubes without anticoagulant and kept at 37°C for 30 minutes,

then isolated the serum by using a centrifuge at 3000 rpm for 15 minutes. Finally, the resulting serum was used to test urea and creatinine levels by using a commercial kit

Assessment of renal TNF α , IL-6, and total antioxidant capacity (TAC)

The frozen kidney portion was divided into small fragments and washed with cold PBS then the tissue was weighed and homogenized with a high-intensity ultrasonic liquid processor in 1:10 W/V phosphate-buffered saline which contains 1% Triton X-100 and 1% of protease inhibitor cocktail. [37].

The homogenates were centrifuged at 3000 rpm for 20 minutes at 4 C and the supernatant was used for determination of TNF α , IL-6, and TAC according to the manufactured company of ELISA kits (Bioassay Technology Laboratory).

Tissue preparation for Histopathology

Renal tissues of the left kidney acquired after the sacrifice of the rats had been washed with cold isotonic sodium chloride solution 0.9% to remove red blood cells or clots then fixed in 10% formalin and processed in paraffin tissue blocks and microscopic section was taken to involve the renal cortex and pelvis. After that, a section of 5-micrometer thickness had been taken from the blocks and stained with hematoxylin-eosin dye. Histopathological changes were estimated for cellular swelling, cytoplasmic eosinophilia, tubular dilation, loss of brush borders, development of protein casts, epithelial cell desquamation into the lumen, inflammatory reaction, cell lysis, and necrosis [38]. The histopathological test was made in 100 to 400X of original magnification. The severity score of tissue damage was examined by a pathologist in a blinded method. The score of histological changes in the kidney was evaluated as 0, normal; 1, damage including < 25% of tubules; 2, damage involving 25-50% of tubules; 3, damage involving 50-75% of tubules; and 4, damage involving 75-100% of tubules previously described [33]

Immunohistochemistry assessment

Immunohistochemistry was performed to assess Bcl-2 and BAX in kidney tissue. 5 μ m paraffin-embedded sections were stained by utilizing the immunostaining

procedure by use Leica Detection systems. The sections were subjected to xylene Deparaffinization, the rehydration step was done by descending alcoholic concentration. Then blockade of peroxidase activity was done by using 3% H₂O₂, while protein blockers were used for non-specific binding sites, then primary antibody monoclonal antibody against Bcl-2 (Bioassay Technology Laboratory. Cat: BT-MCA0125) (1:100 dilution) and monoclonal BAX antibody (Bioassay Technology Laboratory) (1:100 dilution) were incubated 4C overnight. After washing, the slides were incubated for 1 hr, and then the addition of secondary antibody (biotinylated antibody) was done at 37C for 30 minutes. Sections washed and subjected to HRP for a half-hour. After that the sections were incubated with fresh chromogen (100 μ l/slide) for 15 minutes, finally, sections were exposed to hematoxylin counterstaining [39]. The protein expression of Bcl-2 or BAX was calculated by composite scoring system Q-score (ranged 0-300) that resulting from multiplying the intensity and percent of the staining area. The intensity of stain was scored as 0-3, 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percent of cells stained was graded from 0-100% [40].

Statistical analysis

Statistical analyses were performed using SPSS 26.0 for a window. Inc. Data were expressed as mean \pm SEM. Analysis of variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests. For the histopathological renal changes and the IHC, the Kruskal-Wallis test was used to assess the statistical significance of difference across multiple groups in total severity score (mean score) for histopathological renal changes and mean Q. score for IHC. In all tests, P \leq 0.05 was considered statistically significant.

RESULTS

Influence of vinpo on renal function (serum urea and creatinine)

The levels of S. urea and S.Cr.in control and vehicle groups were significantly higher than levels of sham group, meanwhile, the S. urea and S. Cr. of pretreatment vinpo group were significantly lower than control and vehicle groups, figure (1) and (2).

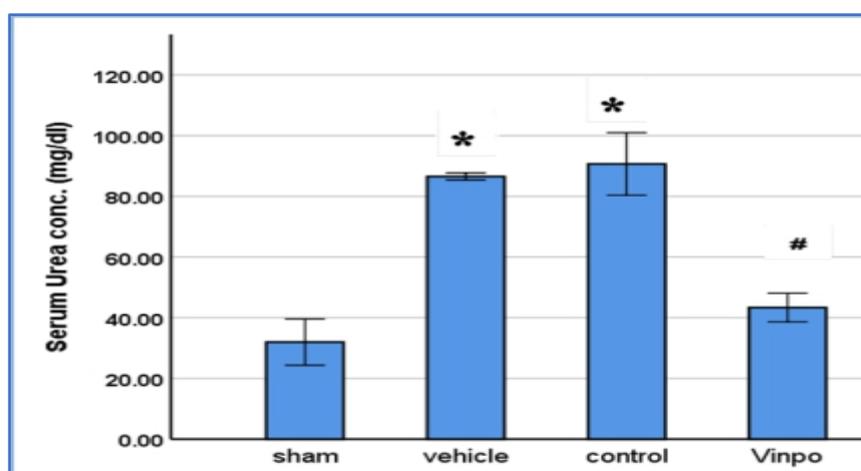


Figure 1. Mean level of serum urea (mg/dl) of all four groups (N = 5): Data are expressed as mean \pm SEM, *P < 0.05 versus sham, #P < 0.05 versus control and vehicle groups.

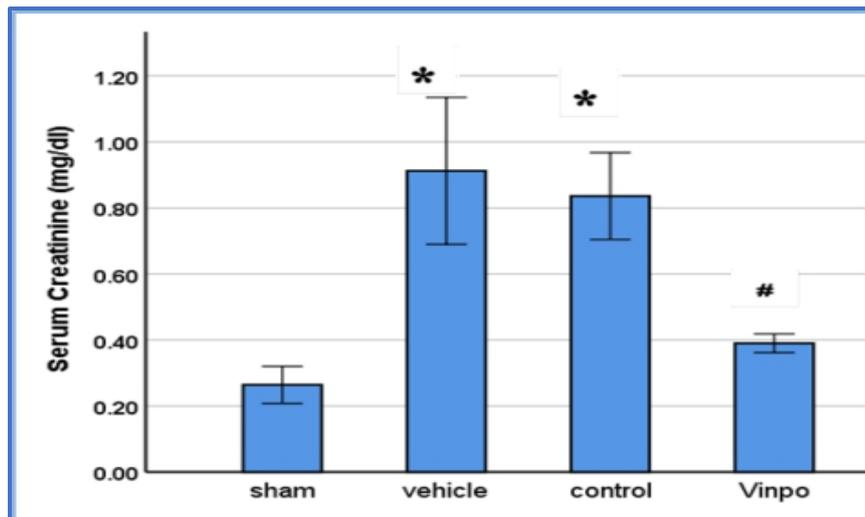


Figure 2. Mean level of serum creatinine (mg/dl) of all four groups (N = 5): Data are expressed as mean \pm SEM, *P <0.05 versus sham, #P <0.05 versus control and vehicle groups

Influence of vinpo on renal oxidative stress parameter (TAC)

Rats in control and vehicle groups showed significantly ($p < 0.05$) lower level of renal (TAC) than that in the sham group. On other hand, it is level in the pretreated vinpo group significantly ($p < 0.05$) higher than that in the control and vehicle groups (figure 3).

Influence of vinpo on renal proinflammatory mediators (TNF α , IL-6)

The renal levels of (TNF α , IL-6) in control and vehicle groups were significantly ($p < 0.05$) higher than that in the sham group, while these levels in the pretreated vinpo group were significantly ($p < 0.05$) lower than that in the control and vehicle groups figure (4) and (5).

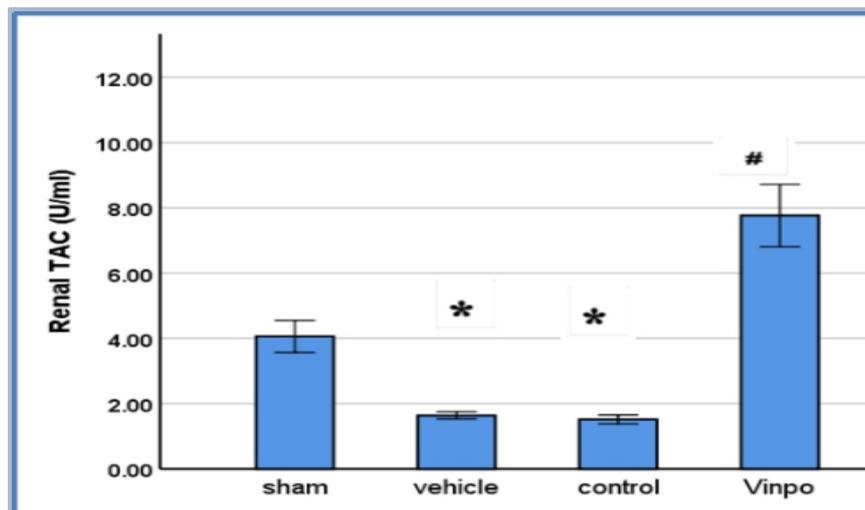


Figure 3. Mean tissue level of total antioxidant capacity (TAC) (U/ml) of all four groups (N = 5): Data are expressed as mean \pm SEM, *P <0.05 versus sham, #P <0.05 versus control and vehicle groups

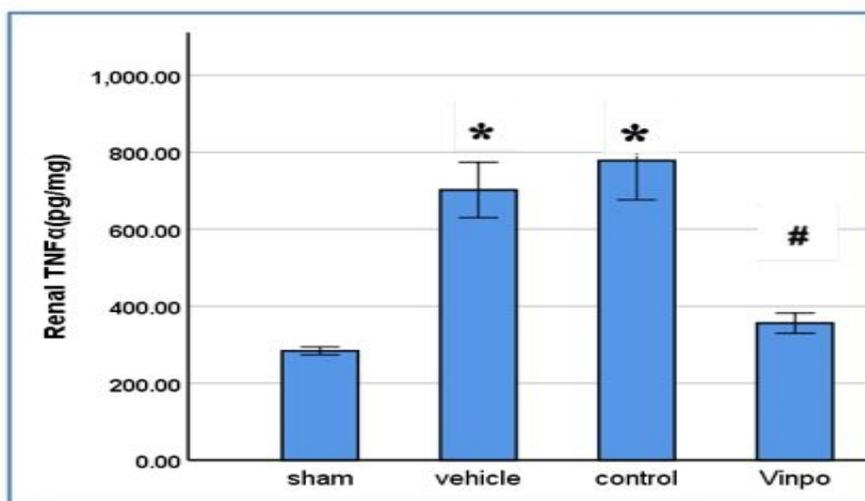


Figure 4. Mean Level of Renal TNF α (pg/mg) of all four groups (N = 5):Data are expressed as mean \pm SEM,*P <0.05 versus sham, #P <0.05 versus control and vehicle groups

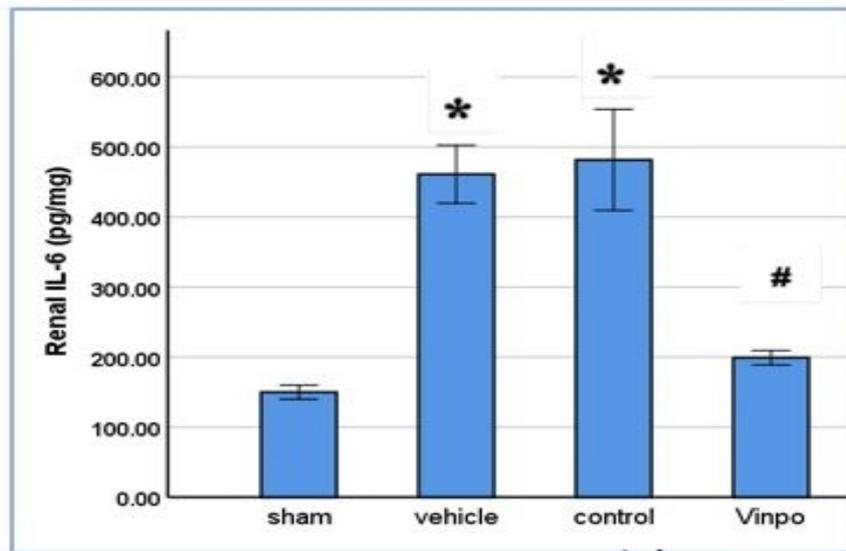


Figure 5. Mean level of Renal IL-6 (pg/mg) of all four groups (N = 5): Data are expressed as mean \pm SEM,*P <0.05 versus sham, #P <0.05 versus control and vehicle groups

Immunohistochemistry results

Influence of vinpo on renal antiapoptotic Bcl-2 IHC stained section

Pretreated vinpo group sections displayed brown

cytoplasmic staining of the BCL-2. Q score of pretreated vinpo groups was seen significantly (p<0.001) higher than the control and vehicle groups while the control and vehicle groups were negatively stained figure (6).

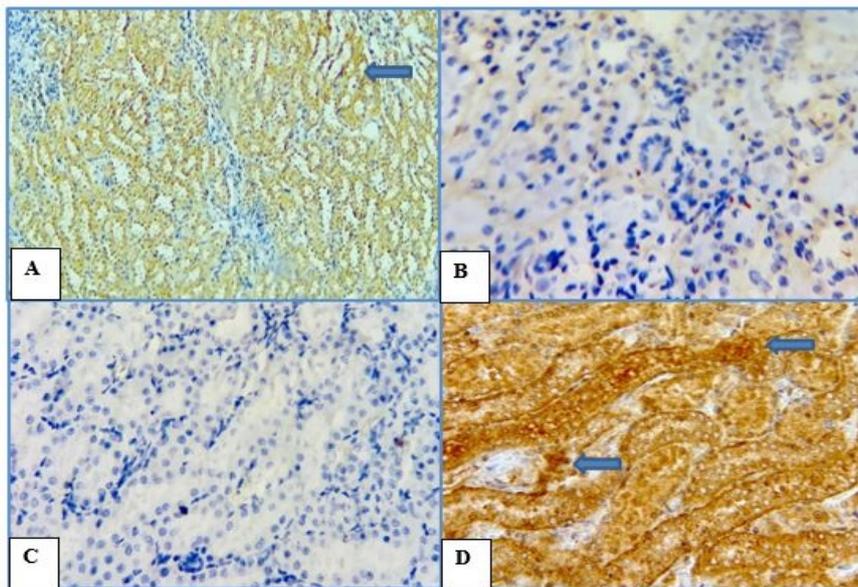


Figure 6. Photomicrographs of sections of rat renal tissue of the (A) Sham group shows positive BCL-2 (brown color) (arrow) X100 (B) Control group negative stain X400 (C) Vehicle group negative stain X400 and (D) Vinpo group showing strong positive stain BCL-2 (brown color) (arrows) X400

Influence of vinpo on renal proapoptotic Bax in IHC stained sections

Pretreated vinpo group sections not displayed staining of the Bax, while the control and vehicle groups section

were positively strong staining in the form of darkly brown stain and seen significantly (p<0.001) higher than the sham group (figure 7).

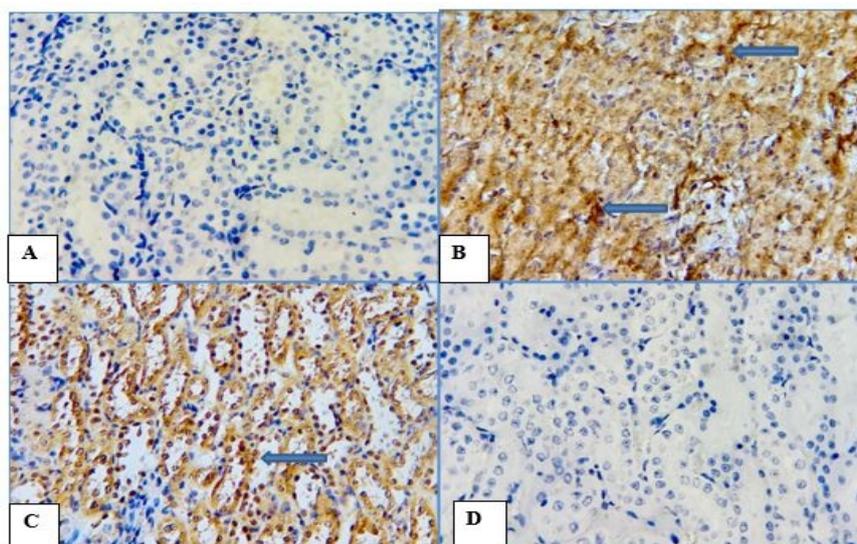


Figure 7. Photomicrographs of sections of rat renal tissue of the (A) Sham group shows negative BAX stain X400 (B) Control group positive stain (brown color) (arrows) X400 (C) Vehicle group positive stain X400 and (D) Vinpo group showing negative BAX stain X400

Histopathological finding

Microscopical examination of H and E sections of control and vehicle groups displayed the development of tubular cell swelling, degeneration of tubular epithelium, congestion of interstitial cytoplasmic eosinophilic besides cast formation, while the renal architecture of sham

group was appeared normal. Whoever the pretreated vinpo group preserved the normal morphology of renal tissue, showing slight swelling of renal tubules cells and less interstitial congestion. The histopathological score of the vinpo pretreated group significantly ($p < 0.001$) lower than the control and vehicle groups figure (8) and (9).

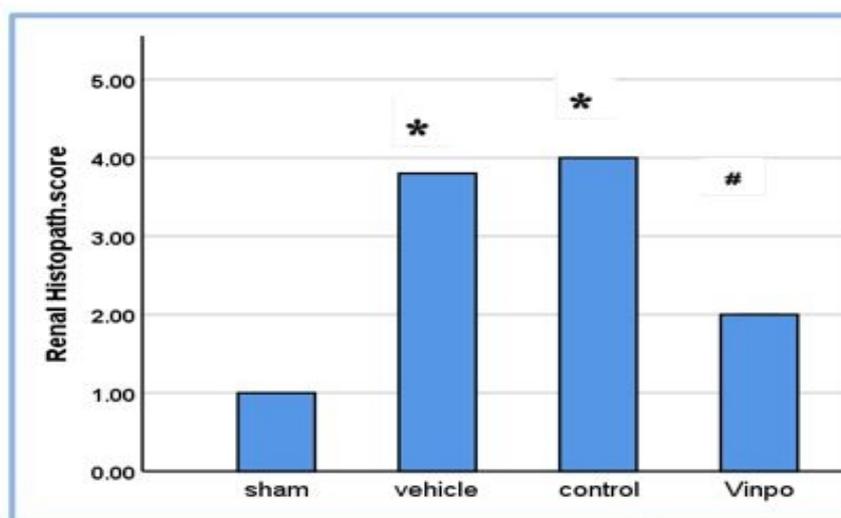


Figure 8. Renal histopathology mean H.score of all four groups (N = 5) $P < 0.001$ versus sham, # $P < 0.001$ versus control and vehicle groups

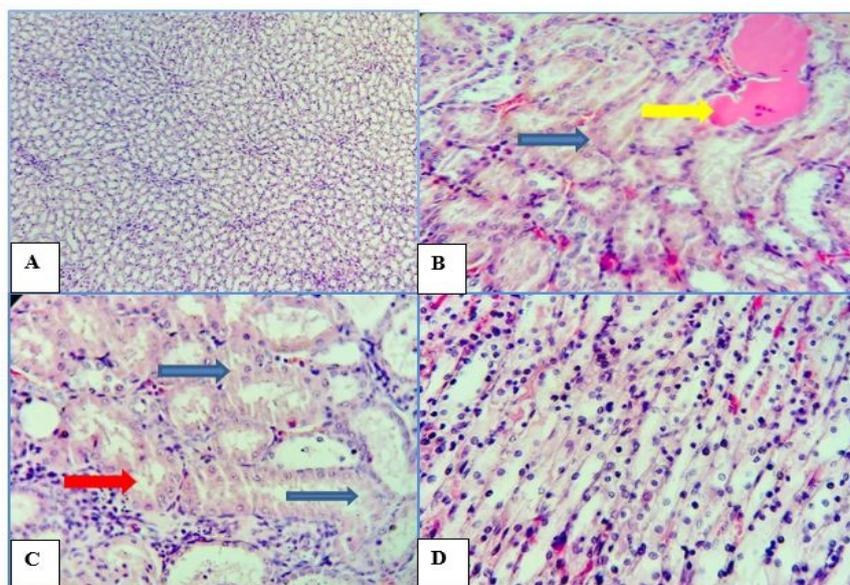


Figure 9. Photomicrographs of sections of left rat kidney tissue with H&E stain of (A) the sham group shows normal histology X100 (B) Control group shows cellular swelling with increased cytoplasmic eosinophilia (blue arrow), eosinophilic cast (yellow arrow) X400 (C) Vehicle group shows cellular swelling, with cytoplasmic eosinophilia (blue arrows), interstitial inflammation (red arrow) X400 (D) Vinpo group showing slight ischemic change and normal histology of renal tubules X400

DISCUSSION

IRI represents the crucial cause of acute kidney injury moreover, IRI is known to be frequently challenging in many clinical conditions such as in transplantation surgery, renal artery stenosis, aortic bypass, sepsis, cardiopulmonary bypass, trauma, urological operations [41]. The renal IR model, which was formed by applying 30 min bilateral renal ischemia followed by reperfusion for 2 h, showed increased oxidative stress and inflammatory response, impaired renal function, decreased antioxidant levels, histopathological renal damage, and increased expression proapoptotic mediator and decrease antiapoptotic protein with the immunohistochemical method. In this study, we showed the protective effects of vinpocetine on renal ischemia-reperfusion injury by investigating inflammatory cytokines, oxidative stress, histopathology of the kidney, and apoptotic mediators' expressions.

Effect of Vinpocetine on renal function parameters (Urea and Creatinine)

The current study showed that urea and creatinine levels were significantly ($P < 0.05$) lower in the Vinpocetine pretreated group when compared to the control and vehicle groups. This result agrees with Kuraishy et al. (2019) [42], this reduction of urea and creatinine may establish the reno-protective effect of Vinpocetine, Wadie and El-Tanbouly (2017) who indicated the vinpo pretreated in inducible diabetic nephropathy rats models improve kidney function and decrease urea and creatinine level also increase creatinine clearance [43]. Fattori et al. (2017) Presenting the Nephroprotective effect of Vinpocetine in diclofenac induced acute kidney injury in rats by decrease urea and creatinine in a dose-dependent manner in addition to alleviate oxidative stress, cytokine production, and cellular apoptosis [44].

Effect of Vinpocetine on the inflammatory mediators (TNF α and IL-6)

The present study demonstrated that renal TNF- α and IL6 levels in the Vinpocetine pretreated group were

significantly lower than levels of the control and vehicle groups. These results seem to be consistent with Fattori et al. (2017), who state the protective effect of vinpo when administered after 30 min of induced kidney injury in mice model by decreasing IL-6 and TNF- α [44]. These findings can be explained due to the inhibitory effect of Vinpocetine treatment on NF- κ B, control the gene expression and production of these inflammatory cytokines [44]. On the other hand, the vinpo decrease the inflammatory response as a result of vinpo ability to decrease oxidative stress which links to increase the production of IL-1 β and TNF- α and extend damaging effects of inflammatory cells [29]. Another proposed mechanism for the anti-inflammatory effect of Vinpocetine may be mediated by the TLR4/MyD88/NF- κ B signaling pathway [45].

Effect of Vinpocetine on Total Antioxidant Capacity (TAC), oxidative stress and ROS formation

The current study demonstrated a significantly higher TAC in the pretreated Vinpocetine groups in comparison to control and vehicle groups, these findings agree with Colombo et al. (2018) [28], which display the Vinpocetine act as a powerful antioxidant modulator by scavenging ROS and decrease lipid peroxidation. These results are in line with those of previous studies [43] illustrated that Vinpocetine decreases lipid peroxidation and increases GSH in renal tissue in a rat model of diabetic nephropathy. Also, Ansari and his colleagues observed that vinpo restored antioxidant capacity by increase TAC, SOD, and GSH in myocardial infarction in a rat model [46]. Also, these outcomes consistent with a recent experimental study by Ristic et al. (2020), that states the alleviations of oxidative status by vinpo [47].

Effect of Vinpocetine on apoptotic markers (Bcl-2 and BAX)

The present study revealed there is a significantly higher level of Bcl2 and lower level of Bax in the vinpo pretreated group as compared to the control and vehicle groups and these results consistent with Zhao et

al.(2020)[34].

Nivison-Smith et al.(2017) declare in the ex-vivo study that Vinpocetine pre-treatment can protect the retina against ischemic insult by reduced cation channel permeability[48]. Also Gabryel et al., 2002 show a decreasing apoptosis marker caspase 3 by metabolic modulation to increase intracellular ATP. The previous study on renal IR has shown that oxidative stress and inflammatory response could activate proapoptotic protein [5]. Therefore, the vinpo could give an antiapoptotic effect by decrease caspase3, Bax, and improve antiapoptotic Bcl-2 proteins through antioxidant ability and anti-inflammatory properties [27-28]. The PI3K/AKT pathway may represent another suggestive mechanism of the Vinpocetine effect while this pathway is important antiapoptotic cellular signaling that may be specifically modulated Connexin (Cx) channels[34]. Connexin regulates inflammasome activation in AKI by controlling oxidative status [50]

Effect of Vinpocetine on renal parenchyma

The microscopic examination finding of the present study displays a significantly lower degree of tissue injury in the vinpo pretreated group in comparison with control and vehicle groups. This result reported by previous studies. The Vinpocetine treatment significantly reduced mice cerebral infarct volumes and neurological scores [45]. Another study reported that Vinpocetine-treated mice showed mild tubular cell vacuolation, a marked reduction in the glomeruli damage, tubular dilatation, and loss of the brush border in gentamycin-induced acute kidney injury[44]. It is possible to Vinpocetine-antioxidant effect as a study of [51] that has been concluding ascorbic acid provides a ROS scavenger action and increased GSH level to stabilize the lysosomal membrane resulting that preserve kidney tissue from lysis.

Abbreviations

AKI (acute kidney injury)
 BAX (Bcl2 Associated X Protein)
 Bcl-2 (B-Cell Lymphoma-2)
 DMSO (dimethyl sulfoxide)
 IL-6 (Interleukin -6)
 IRI (ischemia-reperfusion injury)
 ROS (reactive oxygen species)
 (cGMP) Cyclic guanosine monophosphate
 (cAMP) Cyclic adenosine monophosphate
 vinpo (Vinpocetine)
 S. urea and S. Cr. (serum urea and serum creatinine),
 TAC (total antioxidant capacity),
 TNF α (tumor necrosis factor-alpha),
 NF- κ B (Nuclear factor-kappa B)

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