Journal of Umm Al-Qura University for Medical Sciences

Journal homepage: https://uqu.edu.sa/en/mj

Research Article

Vinpocetine Attenuates 5-Fluorouracil-Induced Renal Intoxication by Regulating Nrf2/ARE, NF-κB/TLR4, and NLRP3/ASC/Caspase-1 Signals in Rats

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ARTICLE INFO

Received: 01/10/2023
Revised: 15/11/2023
Accepted: 01/01/2024

Keywords:
FU-induced nephrotoxicity, Vinpocetine, 5-Flurouracil, NF-κB/TLR4, Nrf2/ARE and NLRP3/ASC/Caspase-1.

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ABSTRACT

Background: One of the most commonly used anti-cancer medications for various tumors is 5-fluorouracil (5-FU). However due to the accompanying nephrotoxicity, its clinical use is restricted. Vinpocetine (VNP) is a derivative of vincamine alkaloid used to treat cognitive disorders and cerebrovascular diseases.

Materials In this study, 40 adult male Wistar rats were randomized into five groups, untreated animals (control), treated with VNP (20mg/kg), 5-FU (30mg/kg), 5-FU+VNP (10mg/kg), and 5-FU+VNP (20mg/kg). The results of all groups were compared to 5-FU rats.

Results: VNP improved renal function by reducing serum urea, creatinine, and NGAL levels while it increased the serum level of albumin. VNP restored the oxidant-antioxidant balance of renal tissues mediated by increasing Nrf2/HO-1 expression. VNP suppressed the inflammation by decreasing MPO and NO2 and reducing IL-1β, TNF-α, and IL-6 levels mediated by suppressing TLR4 and NF-κB expression. Moreover, VNP downregulates NLRP3, ASC, and cleaved caspase 1. Histological analysis of kidney tissues validated our findings. Of note, VNP's Reno protective effects were dose-dependent.

Conclusions: Our data suggest that co-treatment of VNP with 5-FU is a promising agent for mitigating 5-FU-induced nephrotoxicity by NF-κB/TLR4, Nrf2/ARE, and NLRP3/ASC/Caspase-1 signals.

INTRODUCTION

5-Fluorouracil (5-FU) is a drug that inhibits thymidylate synthase, which disrupts the intracellular deoxynucleotide pools necessary for DNA replication (Diasio & Harris, 1989; Longley et al., 2003). 5-FU is used in treating several cancers, including skin, liver, stomach, colorectal, and cancers of the genitourinary system (Longley et al., 2003). Unfortunately, its clinical application is constrained due to the accompanying adverse effects of 5-FU, including nephrotoxicity, hepatotoxicity, and cardiotoxicity (Papanastasopoulos & Stebbing, 2014). Taken into consideration, that oxidative injury, inflammation, and apoptosis play a key role in chemotherapy-evoked nephrotoxicity. Several studies have focused on utilizing antioxidative and antiapoptotic agents to lessen or completely eradicate the nephrotoxicity associated with chemotherapy (Ali, Hassanein, et al., 2021; Hassanein et al., 2021; Rashid et al., 2014).Interestingly, the nuclear factor erythroid 2-related factor2 (Nrf-2)-Kelch ECH associating protein 1 (Keap-1) signal is activated as a result of free radicals disrupting the oxidant-antioxidant balance (Bellezza et al., 2018).

This pathway is crucial for protecting against ROS-induced stress insults and has a vital role in renal protection (Ali, Sayed, et al., 2021; Hassanein et al., 2019; Kamel et al., 2022). In addition, when there is an increase in free radicals, Nrf-2 separates from Keap-1 and moves into the nucleus binds to the ARE, and starts the transcription of detoxifying genes like heme oxygenase-1 (HO-1) (Lu et al., 2016). An excess of ROS can activate several signals, such as NF-κB (Zhang et al., 2016), which evokes cytokines release, including IL-1β, IL-6, and TNF-α. According to earlier research, some natural substances can safeguard the liver and kidney by suppressing the NF-κB and upregulating the Nrf2 signal (Caglayan et al., 2018; Hassanein et al., 2020).

Pyroptosis is a form of pro-inflammatory programmed cell death triggered by a member of the caspase family involved in inflammation (Galluzzi et al., 2018). Most

Doi: https://doi.org/10.54940/ms31573092

myeloid-derived phagocytes experience pyroptosis (Vande Walle & Lamkanfi, 2016). With the growth of pyroptosis research in recent years, academics have committed attention to studying pyroptosis in kidney disorders. Pyroptosis likely contributes to kidney disorders. Additionally, some researchers have produced drugs that target kidney-related disorders from the perspective of pyroptosis, which might serve as a suggestion for diagnosis, focused therapy, and prognosis of renal disorders (Zhang et al., 2021).

Vinpocetine (VNP) is a derivative of vincamine alkaloid, an ethyl ester of apovincamine used to treat cerebrovascular illnesses (Bagoly et al., 2007). Through an increase in cerebral blood flow and oxygen consumption, VNP enhances brain metabolism and peripheral circulation (Abdelzaher et al., 2021; Alkuraishy et al., 2014; Khalil et al., 2022). VNPs possess anti-inflammatory and antioxidant properties in different models (Tashkandi et al., 2023; Zhang & Yang, 2014). Consequently, this investigation aims to explore the antioxidant, anti-inflammatory, and anti-pyroptotic activities of VNP in 5-FU-induced renal intoxication.

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats were acclimatised in the animal facility for two weeks before starting the experiments. The animal home was maintained at a humidity level of 50% and a temperature of 25 degrees Celsius. There was a 12-hour cycle of light and dark. There was plenty of normal food and drink. This study's protocol was approved by the Umm Al-Qura University Ethical Committee – under Approval No. (HAPO-02-K-012-2023-12-1919).

Experimental design

In this experiment, 40 adult male Wistar rats were randomised into five groups of eight rats each. They were utilised to administer one of the following daily regimens throughout the study's first ten days:
-Group I: Administered the vehicle (control).
-Group II: Administered VNP (20 mg/kg/day) (Sharma et al., 2022) by gavage.
-Group III: Injected by 5-FU (30 mg/kg/day) (Lokman et al., 2022) for 5 consecutive days and started on the 5th day of the experiment.
-Group IV (5-FU + VNP 10mg): Injected by 5-FU as previously indicated plus VNP (10 mg/kg/day) by gavage.
-Group V (5-FU + VNP 20mg): Injected by 5-FU as previously indicated plus VNP (20 mg/kg/day) by gavage.

Samples and tissue preparation

The drug administration, the rats were sacrificed at the end of the study under ketamine (100 mg/kg i.p.) anaesthesia. Thereafter, one of the two kidneys was homogenised in PBS to yield a 10% w/v homogenate for the biochemical tests. The other one was processed for histological and immunohistochemical examinations.

Kidney function biomarkers

Creatinine, urea, and albumin levels in serum were measured using commercial vendor kits (Spectrum Company, Egypt) to look for nephrotoxicity markers. The renal content of the NGAL was measured using ELISA kits (Elabscience Company, USA) according to the manufacturer's instructions. The developed colour for the assay was read at 450 nm.

Assessment of renal cytokines

Following the manufacturer's instructions, ELISA kits were employed to assess the renal IL-1β, TNF-α, and IL-6 levels.

Measurement of renal oxidative injury parameters

The levels of GSH, MDA, and NO in the kidneys were measured using the techniques outlined by Mihara and Uchiyama (Mihara & Uchiyama, 1978), Ellman (Ellman, 1959), and Montgomery and Dymock (Montgomery & Dymock, 1961), respectively. SOD, GST, and MPO renal enzymatic activities were performed following the procedures previously reported (Keen et al., 1976; Krawisz et al., 1984; Marklund, 1985).

Histopathological assessments

A standard histology protocol was used to fix the kidney specimen in 10% buffered formalin and embed it in paraffin. H&E was used to stain five-micron slices. After that, histopathological examination of kidney tissue lesions was carried out under a light microscope (Inoue et al., 2009).

Immunohistochemical analysis

Paraffin-embedded blocks that were 4-µm thick were used. Blocking was carried out for two hours using 5% BSA in TBS. The sections were then incubated with primary antibodies for TLR4 and NF-κB in TBS at 4°C. After applying a secondary antibody treatment, the sections were washed in TBS. Before incubating in a solution containing DAB and H2O2, the slides were also washed with TBS. Light microscopy was used to examine the sections (Ramos-Vara, 2005).

qRT-PCR analysis

qRT-PCR was used to evaluate Keap-1, Nrf2, HO-1, and GAPDH mRNA levels. Thermo Fisher Scientific's TRIzol reagent was used to isolate total RNA from the kidney tissues (USA). A commercial kit from Vivantis Technologies (Malaysia) was used to create the cDNA, and the SYBR Green master mix was used for the amplification. Normalisation with the housekeeping gene

GAPDH was applied. For data calculations of the expression, the 2−ΔΔCt method was used (Livak & Schmittgen, 2001). The primers used for qRT-PCR are listed in Table 1.

Western blotting

Bradford method was used to determine protein concentration (Bradford, 1976). SDS-PAGE was used to separate the proteins, and they were then transferred to PVDF membranes and blocked with TBST. Antibodies against NLRP3, ASC, cleaved caspase-1, and β-actin were used. The membranes were rinsed three times with TBST before incubation with an ALP-conjugated secondary antibody. The BCIP/NPT kit was used to identify the protein expression after multiple washes. Image J® was used for the quantification of the protein bands. The β-actin was used for normalisation.

Table 1: *qRT-PCR Primers Used for Gene Expression*

Target Genes	Forward primer	Reverse primer	Accession Number	Product Size (bp)
Keap-1	5'-TCAGCTAGAGGCGTACTGGA-3'	5'-TTCGGTTACCATCCTGCGAG-3'	XM_006242591.3	500
Nrf-2	5'-ATTGCTGTCCATCTCTGTCAG-3'	GCTATTTTCCATTCCCGAGTTAC-3'	NM_001399173.1	109
HO-1	5'-TGCTTGTTTCGCTCTATCTCC-3'	5'-CTTTCAGAAGGGTCAGGTG-3'	XM_032887931.1	102
GAPDH-1	5'-TGCTGGTGCTGAGTATGTCG-3'	5'-TTGAGAGCAATGCCAGCC-3'	XM_017592435.1	265

Statistical analysis

The data were analysed using GraphPad Prism 7.0 and presented as mean ± SEM. A one-way ANOVA was used to evaluate group differences, with $P \leq 0.05$.

RESULTS

VNP protects against 5-FU-induced renal injury

In 5-FU-induced kidney injury, creatine (Figure 1A), urea (Figure 1B), and NGAL (Figure 1C) levels were remarkably elevated by 2.62, 2.8, and 9.32-fold, respectively, while the albumin (Figure 1D) level significantly decreased compared to control groups. Intriguingly, VNP in both doses significantly decreased creatine, urea, and NGAL and markedly restored albumin levels relative to the 5-FU-control group dose-dependently (Figure 1).

VNP mitigates 5-FU-induced histological alteration in the kidneys

Kidney sections from the normal control group and VNP (20mg) displayed the normal architecture of renal corpuscle with intact glomerulus and bowman's capsule, proximal convoluted tubules, as well as distal convoluted tubules, as well as distal convoluted tubules. On the other side, kidney sections from rats injected with 5-FU highlighted severe degenerative changes along the renal cortex area. The renal corpuscle presented with a deteriorated bowman's capsule and a vacuolated glomerulus. Renal tubules suffered from seri ous degeneration, loss of their normal structure, necrobiotic changes, and epithelial desquamation.

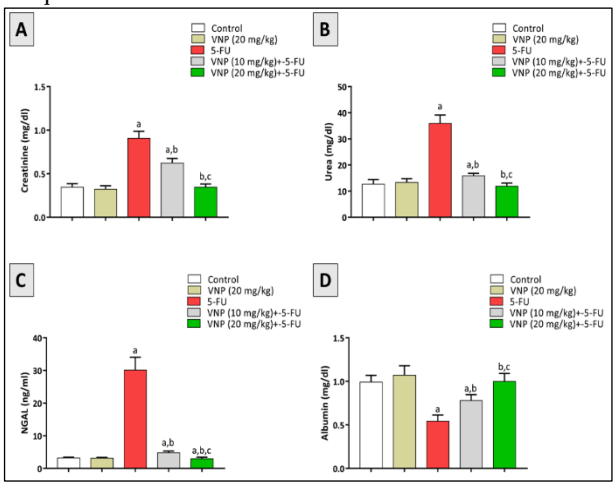

Figure 1: *VNP protects against 5-FU-induced renal impairment. VNP significantly decreased creatine (A), urea (B), and NGAL (C) and markedly restored albumin (D) dose-dependently. The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

Also, interstitial oedema, haemorrhage, and infiltrated inflammatory cells were noticed. In contrast, kidney sections from rats treated with a small dose of VNP emphasised moderate progress along tissue structure. Renal corpuscle existed in normal assembly. Some renal tubules demonstrated obvious necrotic and degenerative changes and epithelial desquamation. Interstitial haemorrhage and aggregated inflammatory cells were detected in a few amounts. A larger dose of VNP underlined great enhancement along tissue structure. Renal corpuscle existed with intact bowman's capsule, RBCs inside glomerulus, and increased area of glomerular space. Renal tubules are marked mostly in normal appearance except for a few with degenerated structures and renal cast inside tubules. Interstitial haemorrhage was also observed (Figure 2).

VNP mitigates oxidative deteriorations induced by 5-FU

5-FU induced lipid peroxidation and decreased the antioxidant status in renal tissues, as evidenced by increasing MDA (Figure 3A) content by 2.01-fold and depleting GSH (Figure 3B), GST (Figure 3C) and SOD (Figure 3D) levels by 50.08%, 53.18%, and 68.57%, respectively. On the contrary, VNP reverted the depletion effect of 5-FU on the antioxidants GSH, GST, and SOD, and VNP inhibited lipid peroxidation, evidenced by lowering MDA contents. Significantly, VNP antioxidant effects in a dose-dependent manner (Figure 3). Impressively, Keap-1 (Figure 4A) was dramatically upregulated by 2.58-fold in the 5-FU-treated group, while Nrf-2 (Figure 4B) and HO-1 (Figure 4C) levels were significantly downregulated by 60.5% and 60.67, respectively.

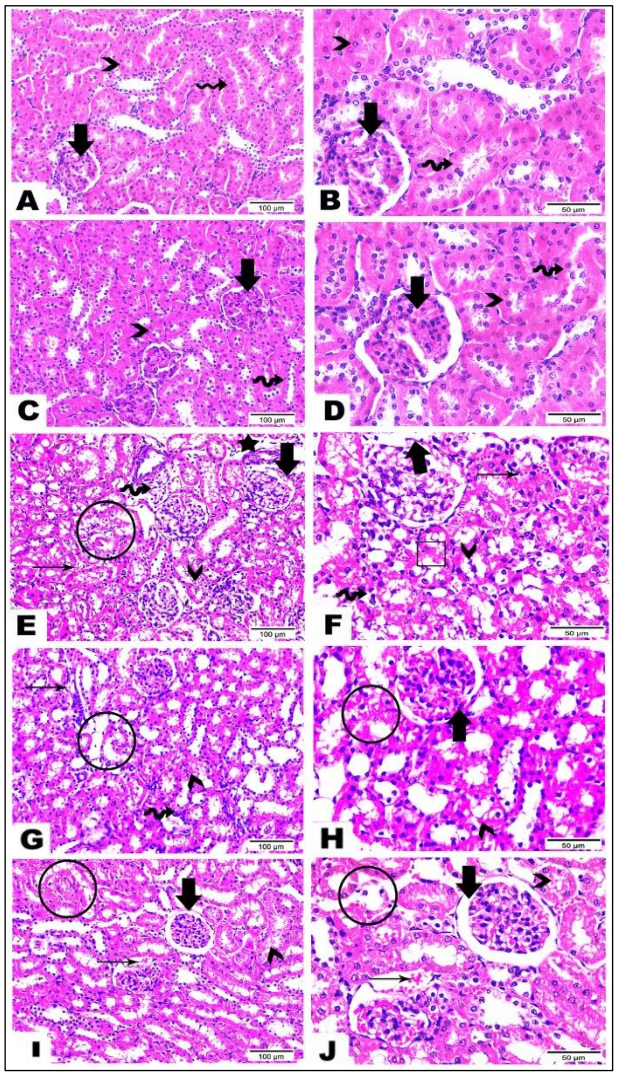

Figure 2: *VNP attenuates 5-FU-induced histological alteration in the kidneys.*

Kidney sections from the normal control group (A & B) and VNP (20mg) (C & D) displayed the normal architecture of renal corpuscle with intact glomerulus and bowman's capsule (thick arrows), proximal convoluted tubules (arrowheads) as well as distal convoluted tubules (wave arrows). On the other side, kidney sections from rats injected with 5-FU highlighted severe degenerative changes along the renal cortex area. The renal corpuscle presented with a deteriorated bowman's capsule and vacuolated glomerulus (thick arrows). Renal tubules suffered from serious degeneration with loss of their normal structure (circle), necrobiotic changes (cube), and epithelial desquamation (arrowhead). Also, interstitial oedema (star), haemorrhage (thin arrow), and infiltrated inflammatory cells (wave arrow) were noticed. In contrast, kidney sections from rats given small doses of VNP emphasised moderate progress along tissue structure. Renal corpuscle existed in normal assembly (thick arrow). Some renal tubules demonstrated obvious necrotic and degenerative changes (circle) and epithelial desquamation (arrowhead). Interstitial haemorrhage (thin arrow) and aggregated inflammatory cells (wave arrow) were detected in a few amounts. A larger dose of VNP underlined great enhancement along tissue structure. Renal corpuscle existed with intact bowman's capsule, RBCs inside glomerulus, and increased area of glomerular space (thick arrow). Renal tubules are marked mostly in normal appearance except a few with degenerated structure (circle) and renal cast inside tubules (arrowhead). Interstitial haemorrhage was also observed (thin arrow). (H&E, Scale Bar=100µm & 50µm).

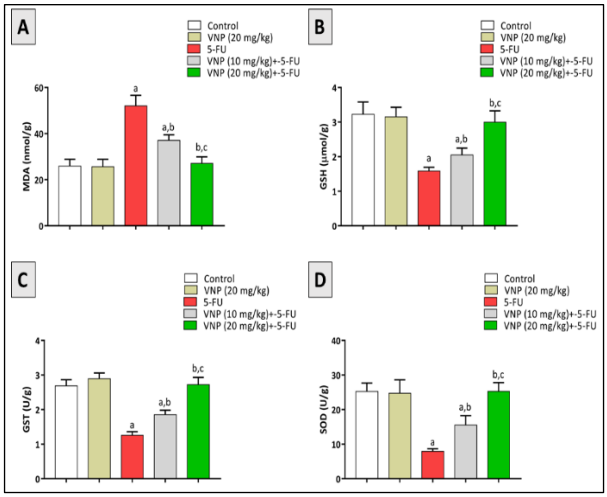

Figure 3: *VNP dampens oxidative deteriorations induced by 5-FU. VNP inhibited lipid peroxidation, evidenced by lowering MDA (A) contents and increased antioxidants GSH (B), GST (C), and SOD (D). The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

However, VNP significantly downregulated Keap-1 dose-dependently while upregulating Nrf-2 and HO-1 expression levels compared to rats given 5-FU alone (Figure 4).

VNP dampens 5-FU-induced inflammation

Additionally, we investigated the anti-inflammatory effect of VNP. 5-FU significantly increased MPO and NO2, by 2.91 and 2.63-folds, respectively, along with higher levels of TNF-α, IL-1β, and IL-6 by 6.67, 7.53, and 4.41-folds, respectively. However, co-treatment with VNP caused a dose-dependent decrease in the renal levels of these biomarkers (Figure 5).

To explore the anti-inflammatory underlying mechanism of VNP, the TLR4 and NF-κB were assessed using immunohistochemistry. The TLR4 (Figure 6A) and NF-κB (Figure 6B) were highly upregulated in renal tissue in the 5-FU control group by 3.27 and 3.54-folds, respectively, while the administration of VNP resulted in a significant and dose-dependent reduction. These data proved the anti-inflammatory activity of VNP by regulating TLR4/NF-κB signal (Figure 6).

Interestingly, the co-administration of VNP attenuated these effects dose-dependently by downregulating NLRP3, ASC, and cleaved caspase-1 (Figure 7). The results prove VNP's anti-pyroptotic effect in the 5-FU kidney model.

VNP attenuates 5-FU-induced pyroptosis

Afterwards, we studied the anti-pyroptotic effect of VNP on 5-FU-induced kidney toxicity by measuring caspase-1, ASC and NLRP3 levels using W.B analysis. In the current investigation, the protein expression level of NLRP3, ASC, and cleaved caspase-1 was notably upregulated in 5-FU control rats by 12.07, 2.72, and 12.92-folds, respectively.

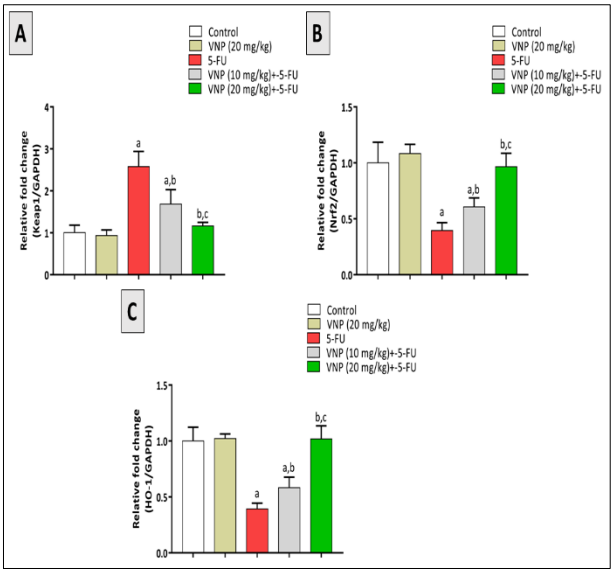

Figure 4: *Effect of VNP on Keap1/Nrf-2/HO-1 signal in kidney injury induced by 5-FU. VNP, dose-dependently, significantly downregulated Keap-1 (A) while upregulated Nrf-2 (B) and HO-1 (C) expression levels. The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

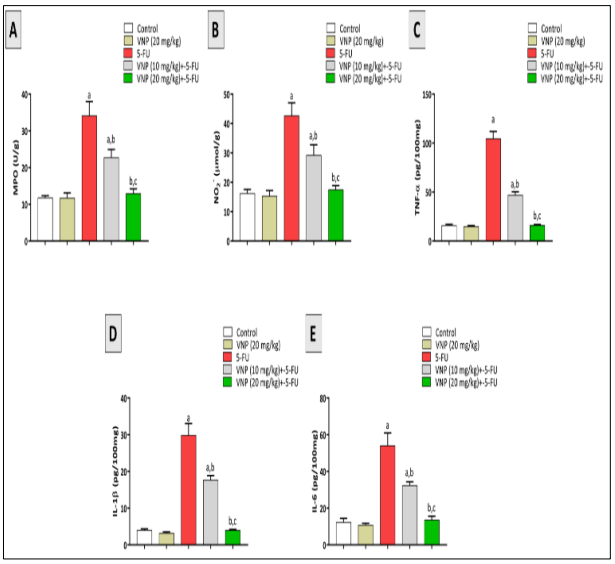

Figure 5: *VNP mitigates 5-FU-induced inflammation. VNP significantly decreased MPO (A) and NO2 (B), along with the decline in the levels of TNF-α (C), IL-1β (D), and IL-6 (E). The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

DISCUSSION

Numerous investigations have shown the key role of ROS/RNS, inflammation, and apoptosis in AKI (Al-Kuraishy et al., 2019). VNP reduces oxidative injury, inflammation, and renal cell death following AKI (Al-Kuraishy et al., 2019; V. Fattori et al., 2017). Hence, the current study was conducted to look into the antioxidants,

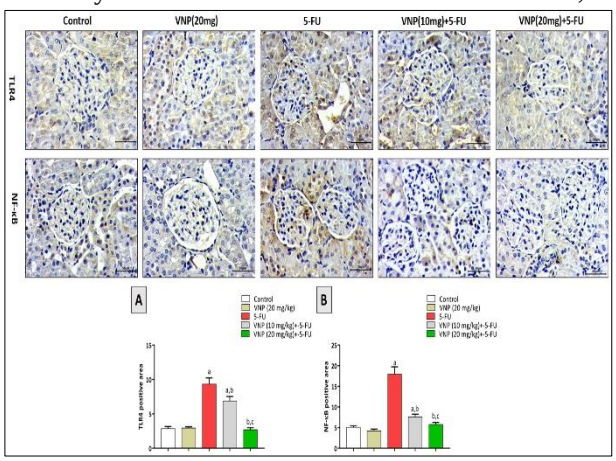

Figure 6: *Effect of VNP on TLR4/NF-κB signal in kidney injury induced by 5-FU. VNP decreased TLR4 (A) and NF-κB (B) dose-dependently. The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

The study explores the anti-inflammatory and anti-pyroptotic effects of VNP against 5-FU-induced kidney injury and the impact of Nrf2, TLR4/NF-κB, and pyroptosis signals on these effects.

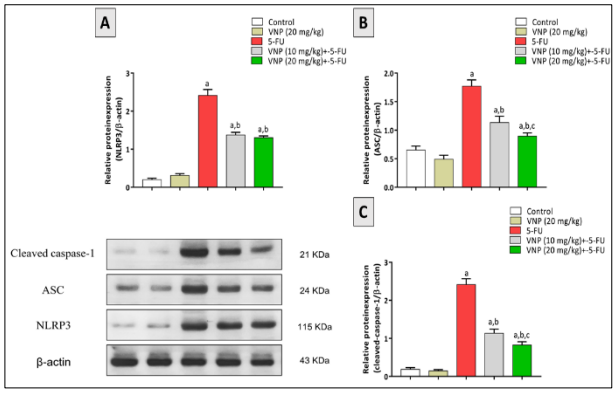

Figure 7. *VNP attenuates 5-FU-induced pyroptosis. VNP downregulated NLRP3 (A), ASC (B), and cleaved caspase-1 (C). The significant differences from the normal, 5-FU, and 5-FU+VNP (10mg) groups are shown in letters a, b, and c, respectively.*

5-Flurouracil is used widely in managing pancreatic, colon, breast, gastrointestinal, head, and neck cancers (Longley et al., 2003). However, it has significant nephrotoxicity that limits its usage (Alvarez-Cabellos et al., 2007; Inoue et al., 2009). The current study showed that

5-FU caused significant renal damage, evidenced by high urea, creatinine, and NGAL, while the albumin levels were decreased. These findings are consistent with several earlier studies (El-Sherbiny et al., 2021; Rashid et al., 2014). NGAL supports renal nephron injury (Paragas et al., 2012). Intriguingly, VNP has renoprotective effects, which were supported by restoring the level of these biomarkers to near that of the normal control rats. Our data are consistent with the VNP's reported ability to reduce acute kidney injury caused by gentamicin (Al-Kuraishy et al., 2019). In addition, VNP reduced 5-FU-induced histopathological changes and kidney damage induced by 5-FU, confirming the obtained biochemical findings.

Emerging evidence indicates that oxidative stress, which causes excessive free radicals and ROS production, contributes to the 5-FU renal damage pathogenesis (Raghu Nadhanan et al., 2012; Rashid et al., 2014). The present data demonstrated that 5-FU induced lipid peroxidation and related cellular membrane damage in addition to cellular GSH, SOD, and GST antioxidant depletion. This outcome is in line with previously reported studies (Abraham et al., 2010; Rashid et al., 2014). Our data also demonstrated that VNP has a crucial role in suppressing oxidative stress via restoring Nrf2 and HO-1 levels and reducing the levels of Keap-1 (Lakshmi & Subramanian, 2014). In line with these findings, Song et al. showed that VNP could protect against cisplatin nephrotoxicity via Nrf2 activation (Arab et al., 2018). Overall, our results indicate that the antioxidant effect of VNP against renal oxidative damage is mediated via the upregulation of Nrf2.

This study revealed an inflammatory response associated with 5-FU injection, proved by increased renal MPO, NO2, TNF-α, IL-1β, and IL-6 levels, consistent with a recent study (Ragab et al., 2014). Evidence suggests that the release of cytokines contributes to the inflammatory response in various renal diseases (Liu et al., 2012). Interestingly, the current study reports the association of NF-κB and TLR4 activation in mediating the damaging inflammatory effects of 5-FU on rat kidneys. The role of NF-κB activation in 5-FU-associated renal impairment has been described in earlier investigations (Arab et al., 2018; Rashid et al., 2013). Interestingly, our study showed that VNP markedly decreased MPO and NO2, as well as renal cytokines. Similarly, VNP proved its ability to inhibit cytokine release in diclofenac-induced kidney injury (Victor Fattori et al., 2017). Furthermore, we discovered that VNP significantly inhibited the TLR4/NF-κB pathway and its downstream cytokines, demonstrating its multifaceted anti-inflammatory effects. In line with this, VNP decreases inflammatory responses by inhibiting the TLR4/NF-κB signal in ischemic brain damage (Wu et al., 2017). As a result, VNP anti-inflammatory mechanism by regulating TLR4/NF-κB axis and their downstream cytokines.

Then to emphasise the anti-pyroptotic renoprotective effect of VNP on 5-FU kidney injury. Our study revealed that 5-FU increased ASC, NLRP3, and cleaved caspase-1. Recent studies revealed that the NF-κB and MAPK signals involved in acute and chronic inflammation may activate the NLRP3 inflammasome (Fann et al., 2018). Inflammasome NLRP3 expression may be decreased by suppressing the NF-κB activity (Shao et al., 2016). Our findings revealed that VNP reduced ASC, NLRP3, and cleaved caspase 1. Similarly, VNP has proven its ability to downregulate NLRP3/NF-κB in ischemic stroke (Han et al., 2020). Taken together, VNP may have anti-pyroptotic activity via the downregulation of NLRP3, ASC, and cleaved caspase 1.

CONCLUSION

Vinpocetine effectively attenuated 5-FU-induced renal intoxication. VNP restored the oxidant-antioxidant balance of renal tissues mediated by upregulating Nrf2/HO-1. VNP suppressed the inflammatory response mediated by decreasing TLR4 and NF-κB expression. VNP attenuates pyroptosis mediated by downregulating NLRP3, ASC, and caspase 1. VNP's Renoprotective effects were dose-dependent. This data suggested that co-treatment of VNP with 5-FU is a suggesting agent for mitigating 5-FU-induced nephrotoxicity by NF-κB/TLR4, Nrf2/ARE and NLRP3/ASC/Caspase-1 signals.

AUTHOR CONTRIBUTION

NA is responsible for designing the study, Performing the experiments, analysing the data, interpreting the results, and writing the manuscript.

DECLARATIONS

Ethical Approval

This study's protocol was approved by the Umm Al-Qura University Ethical Committee (Approval No. HAPO-02-K-012-2023-12-1919).

Participants Consent

Not Applicable.

Source of Funding

Not Applicable.

Conflict of Interest

All authors have declared that no financial support was received from any organisation for the submitted work.

All authors have declared that no other relationships or activities could appear to have influenced the submitted work.

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