



# Involvement of Nrf2/HO-1 antioxidant signaling and NF-κB inflammatory response in the potential protective effects of vincamine against methotrexate-induced nephrotoxicity in rats: cross talk between nephrotoxicity and neurotoxicity

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## Abstract

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent widely used in the treatment of cancer and autoimmune diseases like rheumatoid arthritis. However, its use has been limited by its nephrotoxicity. MTX-induced renal injury results in uremia which may influence both the peripheral and central nervous systems causing cognitive and memory problems. The nephroprotective and neuroprotective activities of vincamine (10, 20 and 40 mg/kg), a natural alkaloid with known anti-oxidant, anti-apoptotic and neuroprotective properties, were investigated against MTX-induced toxicity. MTX treatment increased the markers of kidney injury and relative kidney weight, lipid peroxidation, nuclear factor-κB (NF-κB), inflammatory markers, tumor necrosis factor-α, interleukin-1β, myeloperoxidase and cyclooxygenase-2 and caspase-3 expressions, decreased catalase and superoxide dismutase activities, interleukin-10 and ATP levels and antioxidant proteins, nuclear factor erythroid 2-related factor 2 (Nrf2) and hemeoxygenase-1 (HO-1). Moreover, it disturbed rats' behavior in the locomotor activity test, Y-maze and passive avoidance task. Treatment with vincamine (40 mg/kg) effectively ameliorated MTX-induced renal injury via increasing the expression of Nrf2 and HO-1 suppressing oxidative stress, decreasing the expression of inflammatory markers, NF-κB and caspase-3 pathways and enhancing ATP levels. Additionally, it restored locomotor activity in the locomotor test and memory functions in passive avoidance and Y-maze tests.

**Keywords** Nephrotoxicity · MTX · Vincamine · ROS · Uremic toxins · NF-κB/Nrf2/HO-1 pathway

## Introduction

Kidneys are one of the most important organs for metabolism and elimination of the unnecessary toxic metabolites from the body, as well as for homeostasis, normal electrolyte balance and promoting normal blood pressure within the body (Onopiuk et al. 2015). The kidney injury induced by nephrotoxic drugs is often interpreted as inflammation in the glomerulus, proximal tubules and surrounding cellular matrix. This occurs along with cytotoxicity due to oxidative

stress and increased production of reactive oxygen species (ROS) (Abd El-Twab et al. 2016).

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent which is widely used in the treatment of different types of cancer and autoimmune diseases including rheumatoid arthritis and inflammatory bowel diseases, but its use has been limited by its nephrotoxicity; about 53% of all MTX discontinuations were because of toxicity than because of lack of efficacy 7% (Alarcon et al. 1995). High dose of MTX (HDMTX) is an essential component of the management for different types of malignancies, including acute lymphoblastic leukemia, lymphoma, osteogenic, breast carcinoma, lung carcinoma, and head and neck carcinoma (Ackland and Schilsky 1987; Frei et al. 1980). Since MTX is cleared primarily by renal excretion, the development of acute renal dysfunction induced by HDMTX may occur due to the precipitation of MTX and its metabolites in the renal tubules causing crystal nephropathy or direct tubular toxicity

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via ROS overproduction in the kidney (Dabak and Kocaman 2015).

Several studies have revealed that MTX leads to a reduction in antioxidant enzymatic defense capacity in the blood and renal tissue as well as increased myeloperoxidase (MPO) activity and lipid peroxidation in the renal tissues. Moreover, it has recently been stated that besides oxidative stress, abnormal generation of inflammatory mediators and neutrophil infiltration contribute to MTX-induced renal toxicity and apoptotic histopathological changes (Abdel-Raheem and Khedr 2014; Dabak and Kocaman 2015; Öktem et al. 2006). Renal failure results into uremia which may influence both the peripheral and central nervous systems associated with cognitive and memory problems and may progress into delirium, convulsions, and coma. This may be reversed by hemodialysis and transplantation (Burn and Bates 1998).

Medicinal plants play a central role in managing human diseases, since thousands of years and numerous drugs have been developed from natural sources, e.g., bitter melon extract which has anti-diabetic effect and was used as a treatment for breast cancer. Also, vinca alkaloids have been used clinically for over 40 years (Balunas and Kinghorn 2005; Muhammad et al. 2017). One of them is vincamine, which is a peripheral vasodilator that improves blood flow to the brain and enhances oxygen consumption and glucose utilization. Vincamine is used in a number of brain disorders in the elderly, such as memory disturbances, vertigo, transient ischemic deficits and headache (Fandy et al. 2016). Moreover, vincamine can reduce oxidative stress and exhibit neuroprotective, antioxidant and anti-apoptotic effects (Han et al. 2017). Furthermore, vincamine is considered to be the master compound of cerebrally active alkaloids; its derivatives, for example, 15-methylene-eburnamonine possesses potent anti-cancer activity in several cancer cell lines (Woods et al. 2013).

Studies on the protective effects of vincamine against MTX-induced nephro- and neurotoxicity are rare. Therefore, the current study was designed to explore the possible protective mechanisms of vincamine in kidney injury and the subsequent neurotoxicity induced by MTX in rats.

## Materials and methods

### Animals

Adult male Sprague–Dawley rats, each weighing  $280 \pm 20$  g, were purchased from the Nile Pharma pharmaceutical company, Cairo, Egypt. They were kept in the animal house on a 12 h light/dark cycle, with free access to standard rodent chow and water provided ad libitum under constant temperature ( $25 \pm 2$  °C) and humidity (60–70%). Animals were acclimatized to the laboratory conditions for 2 weeks prior

to the experiment. Animal treatments were strictly in adherence to the international and institutional ethical guidelines concerning the care and use of laboratory animals. All experimental protocols were approved by Ain Shams University, Faculty of Pharmacy Ethical Committee for laboratory animal care in research (Memorandum no.150). All efforts were made to minimize animal suffering and reduce the number of animals used.

### Drugs and chemicals

Vials of MTX Injection, USP 2 ml (25 mg/ml), were purchased from Mylan Company (Cairo, Egypt) and injected intraperitoneally (i.p.) in a single dose of 20 mg/kg (Asci et al. 2017). Vincamine was obtained from GlaxoSmithKline Company (Cairo, Egypt), suspended in 1% Tween 80 and then administered orally in three doses of 10, 20 and 40 mg/kg.

Kits for serum blood urea nitrogen (BUN) (cat# UR 2110), superoxide dismutase (SOD) (cat# SD 25 20), catalase (CAT) (cat# CA 251) and malondialdehyde (MDA) (cat# MD 2528) were purchased from Biodiagnostic (Egypt). Serum creatinine (Cr.) (cat# CRE106100) and total protein (cat# TP116250) kits were purchased from Biomed Diagnostics (Egypt). Rat IL-1 $\beta$  PicoKine™ ELISA Kit (cat# MBS175941) was obtained from Boster Biological Technology (USA). Tumor necrosis factor-alpha kit (TNF- $\alpha$ ) (cat# ELR-TNF- $\alpha$ ) was obtained from RayBiotech (USA), and interleukin-10 (IL-10) Quantikine® ELISA Kits R&D Systems (cat# R1000) was purchased from Bio-Techne® company (Minnesota). Adenosine triphosphate (ATP) kit (cat# A22066) was obtained from Molecular Probes Company (USA). Western blot for protein detection kits was purchased from Thermo Fisher Scientific Inc. (USA). Finally, RNA amplification SYBR Green I and reverse transcription polymerase chain reaction (RT-PCR) kit were purchased from Thermo Fisher Scientific Inc. (USA).

### Experimental design

A total of 39 male rats were used in the experiment. To fulfill the goals of the present study, the following design was adopted:

*Part-A:* a dose range-finding study was conducted using 15 rats to select the optimal oral dose of vincamine for the treatment of nephrotoxicity induced by MTX in rats. The animals were divided into five groups ( $n=3$ ) as follows. Group I served as normal control group and received 1% Tween 80 orally. Group II received MTX (20 mg/kg, i.p., single dose) on day 6 of the experiment (Abdel-Raheem and Khedr 2014; Yuksel et al. 2016). Groups III, IV and V received MTX (20 mg/kg, i.p., single dose) and were treated with vincamine (10, 20 and 40 mg/kg, orally, respectively)

(Abdel-Salam et al. 2015; El-Dessouki et al. 2018) for 10 successive days 5 days before and continuing 5 days after MTX. On the last day of the experiment, blood samples were collected via the retro-orbital sinus of all rats using heparinized capillary tubes for estimation of creatinine (Cr.) and blood urea nitrogen (BUN) concentration. Following blood collection, animals were weighed in grams, euthanized by cervical dislocation and then decapitated. The kidneys were rapidly removed, washed with saline, dried between two filter papers and weighed in grams for the assessment of the relative kidney weight. These parameters along with the histopathological examination were used for the assessment of the optimal nephroprotective dose of vincamine against MTX-induced nephrotoxicity.

**Part-B:** with regard to the results obtained from the dose range-finding study, the best effective dose of vincamine was selected for further investigation. The animals were divided into four groups ( $n=6$ ) as follows. Group I served as the normal control group and received 1% Tween 80 orally. Group II received vincamine alone (40 mg/kg, orally) for 10 successive days. Group III received MTX (20 mg/kg, i.p., single dose) and Group IV received MTX (20 mg/kg, i.p., single dose) and was treated with vincamine (40 mg/kg, orally) for 10 successive days. On the last day of the experiment, animals were tested for behavior changes, 6 h after the last injection. Following the behavioral tests, animals were weighed in grams, euthanized by cervical dislocation and then decapitated. One kidney together with the brain were excised and rapidly fixed in 10% formalin solution for the preparation of paraffin blocks and used for histopathological and immunohistochemical examination. The other kidney was divided into two parts: the first part was homogenized in ice-cold saline to prepare 10% (w/v) homogenate in 0.1 M phosphate buffer (pH 7.4) and used for the estimation of renal TNF- $\alpha$ , IL-10, IL-1 $\beta$ , SOD, CAT, ATP, MDA, MPO and total protein contents. The second part was placed in radioimmunoprecipitation assay (RIPA) buffer for western blot assessment of caspase-3, NF- $\kappa$ B, Nrf2, and HO-1.

## Behavioral experiment

### Locomotor activity

A box “open field” (68 × 68 × 45 cm) equipped with 15 infrared (IR) beams (wave length = 875 nm and diameter = 0.32 cm), spaced 2.65 cm apart, was placed under the cage. Interruption of IR beams is used as a measure of the animal movements in the cage. A monitor (OptoVarimex-Mini Model B, Columbus Instruments, Ohio, USA) was connected to the apparatus to display the total activity of the animals. The total locomotor activities of the animals are expressed as counts/5 min (Ali et al. 2018).

### Step-through passive avoidance apparatus

The apparatus consists of a passive avoidance box (UGO Basile, Comerio, Italy). It is divided into white lighted compartment and a dark one with steel-rod grid floor wired with a constant current shock generator. The two chambers are separated by an automatically operated sliding gate. Rats were subjected to a training session before treatments of day 9 when they were placed in the light compartment. After 20 s the door was opened, and once the animal stepped through to the dark compartment on its four paws, it received a foot shock of 0.5 mA for 2 s. Rats that failed to step through within a cutoff time of 120 s were excluded. Twenty-four hours later, each rat was gently placed in the light compartment and the latency to step through to the dark compartment was recorded as a passive avoidance behavior indicating memory acquisition, with a cutoff time of 120 s. No electric shock was delivered during test sessions (El-Agamy et al. 2018).

### Y-maze apparatus

The apparatus consists of a black wood maze with three similar opaque arms (40 cm length, 15 cm height and 8 cm width) intersected at 120° and labeled as either arm A, B or C. The animal was positioned in the start arm B and permitted to explore the three arms for 5 min. A valid entry was recorded manually when all the four paws were inside the arm. A spontaneous alternation was counted if the rat had entered the three different arms sequentially. The % of spontaneous alternation was analyzed according to the following formula:  $[(\text{number of alternations})/(\text{total number of arm entries} - 2)] \times 100$  (Ghafouri et al. 2016).

### Estimation of serum creatinine, BUN, renal SOD, CAT, MDA and total protein content

Serum BUN, renal SOD, CAT and MDA contents were assessed by the method described by Fawcett and Scott (1960), Nishikimi et al. (1972), Aebi (1984) and Ohkawa et al. (1979), respectively, using bio-diagnostic kits and were expressed as mg/dl, U/mg protein, U/mg protein and mmol/mg protein, respectively. Serum creatinine and total protein concentration were assessed by the method described by Henry et al. (1974) and Vassault et al. (1986), respectively, using Biomed diagnostic kits and expressed as mg/dl. All the procedures were performed according to the manufacturer's instructions.

### Evaluation of renal IL-1 $\beta$ , TNF- $\alpha$ and IL-10

IL-1 $\beta$ , TNF- $\alpha$  and IL-10 were measured using a procedure similar to that documented by March et al. (1985), El-Agamy

et al. (2018) and Ouyang et al. (2011), respectively, using ELISA kits and expressed as pg/mg.

### Estimation of renal MPO content

The process is based on estimating the hydrogen peroxide-dependent oxidation of *o*-dianisidine catalyzed by MPO, which results in the formation of a complex detected spectrophotometrically at 460 nm (Bradley et al. 1982). It was expressed as U/mg protein.

### Determination of the renal contents of ATP

Renal ATP content was determined according to the procedures similar to those documented by Karamohamed and Guidotti (2001) using Molecular Probes kit and expressed as nmol/mg protein.

### Western blot analysis of caspase-3, NF- $\kappa$ B, Nrf2 and HO-1

About 600 mg of kidney tissue was homogenized with 300  $\mu$ l RIPA buffer using a T-10 Basic Ultra Turrax Homogenizer (IKA). Lysates were centrifuged at 16,000 rpm at 4 °C for 30 min and protein concentrations were estimated with a Bradford Protein Assay Kit (SK3041). 20  $\mu$ g protein concentration of each sample was loaded with an equal volume of 2 $\times$  Laemmli sample buffer at 95 °C for 5 min to ensure denaturation of protein before loading on polyacrylamide gel electrophoresis (Gallagher 2007). Gels were run for 20 min at 50–150 V in an electrophoresis chamber, to allow protein migration and separation in the resolving layer.

After electrophoresis, proteins were transferred to nitrocellulose membrane and the blots were blocked with Tris-buffered saline with Tween 20% (TBST) and 3% bovine serum albumin (BSA) at room temperature for 1 h. The membranes were washed three times with TBST and incubated overnight with the primary antibodies: caspase-3, Phospho-NF- $\kappa$ B p65 (Ser276) polyclonal antibody, Nrf2, HO-1, and  $\beta$ -actin (1:2000 dilution; Thermo fisher, USA). These were rinsed five times each in TBST. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit-IgG, was incubated together with the membrane overnight on a roller shaker at 4 °C. Again, the membrane was washed five times for 5 min with TBST. Detection of proteins bound by the antibody of interest was accomplished by chemiluminescent signals which were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins versus control sample after normalization by  $\beta$ -actin on the Chemi Doc MP imager.

### Histopathological examination of kidney and brain tissues

Kidney and brain samples previously fixed in formalin were washed with tap water and then dehydrated by serial dilutions of alcohols (methyl, ethyl and absolute ethyl). Specimens were cleared in xylene and fixed in paraffin at 56 °C in a hot air oven for 24 h. Paraffinized tissue blocks were prepared for sectioning at 4  $\mu$ m thickness by a sledge microtome. The obtained tissue sections were gathered on glass slides, deparaffinized and stained by H&E for examination using the light electron microscope (Banchroft et al. 1996).

### Immunohistochemical assay of COX-II

Immunohistochemical staining of COX-II was performed using rabbit polyclonal IgG (cat# RB-9072-R7), purchased from Thermo Scientific Co. (USA), according to the manufacturer's directions and protocol (Abdel-Maged et al. 2018). Five random non-overlapping fields per tissue section were analyzed for the calculation of area percent (A %) of COX-II expression of using full HD microscopic camera operated by Leica application module for tissue section analysis (Leica Microsystems GmbH, Wetzlar, Germany). The optical density (OD) of the stained regions in immunostained tissue sections was quantified using image analysis software (Image J, 1.46a, NIH, USA).

### Gene expression analysis of Nrf2 and HO-1 by quantitative real-time polymerase chain reaction

Total RNA was extracted from samples of kidney tissue homogenate using SV total RNA Isolation System (Promega, Madison, USA) according to the manufacturer's instruction.

The isolated RNAs obtained were determined spectrophotometrically at 260 nm. The cDNA was synthesized from 1  $\mu$ g RNA using RT-PCR kit as described in the manufacturer's protocol (cat#18080051, Thermo Fisher Scientific Inc., USA). Real-time PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific Inc., USA). Real-time PCR was carried out with the following conditions: initial amplification, 2 min at 50°, 10 min at 95° and 40 cycles of denaturation for 15 s and annealing/extension at 60° for 10 min. Data from real-time qPCR were analyzed using an Applied Biosystems StepOne™, California, USA, with software version 3.1. All values were normalized to the control housekeeping gene  $\beta$ -actin. The primer sequences are as follows:

Genes	Sequences of PCR primer pairs
Nrf2	F: 5'-ATCCAGACAGACACCAGT GGATC-3' R: 5'-GGCAGTGAAGACTGAACT TTCA-3'
HO-1	F: 5'-TGCTCAACATCCAGCTCT TTGA-3' R: 5'-GCAGAACTTGCACCTTG TTGCT-3'
$\beta$ -actin	F: 5'-TGTTTGAGACCTTCAACACC-3' R: 5'-CGCTCATTGCCGATAGTG AT-3'

## Statistical analysis

Non-parametric data were expressed as medians and interquartile range and analyzed by Kruskal–Wallis test followed by Dunn' test as a post hoc test.

Parametric data were expressed as mean  $\pm$  SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. All statistical analyses were performed using the GraphPad Prism software (version 6.01, Inc., 2012, La Jolla, CA, USA). Probability values of less than 0.05 were considered statistically significant.

## Results

### Screening the nephroprotective dose of vincamine against MTX-induced nephrotoxicity

#### Effects of vincamine on relative kidney weight, serum Cr. and BUN levels of MTX-treated rats

One-way ANOVA statistical analysis showed significant differences among groups on the percent changes in relative kidney weight, serum Cr., and serum BUN [ $F(4, 10) = 7.88$ ,  $P < 0.001$ ,  $F(4, 10) = 14.06$ ,  $P < 0.001$  and  $F(4, 10) = 21.78$ ,  $P < 0.001$ , respectively] as shown in Fig. 1. Rats that received MTX (20 mg/kg, I.p single dose) on the 6th day of the experiment showed a significant increase in relative kidney weight, serum Cr. and serum BUN by 24.45%, 48.54% and 87.46%, respectively, as compared to that of the normal control group. Concomitant treatment with vincamine (10 mg/kg, p.o) for 10 days caused a non-significant decrease in relative kidney weight, serum Cr. and serum BUN by 11.71%, 13.68% and 20.96%, respectively, as compared to the MTX group. Concomitant treatment with vincamine (20 mg/kg, p.o) for 10 days caused a significant decrease in relative kidney weight, serum Cr. and serum BUN by 16.77%, 22.76% and 26.99%, respectively, as compared to the MTX group. Concomitant treatment with

vincamine (40 mg/kg, p.o) for 10 days caused a further significant decrease in relative kidney weight, serum Cr. and serum BUN by 20.93%, 36.14% and 34.53%, respectively, as compared to the MTX group.

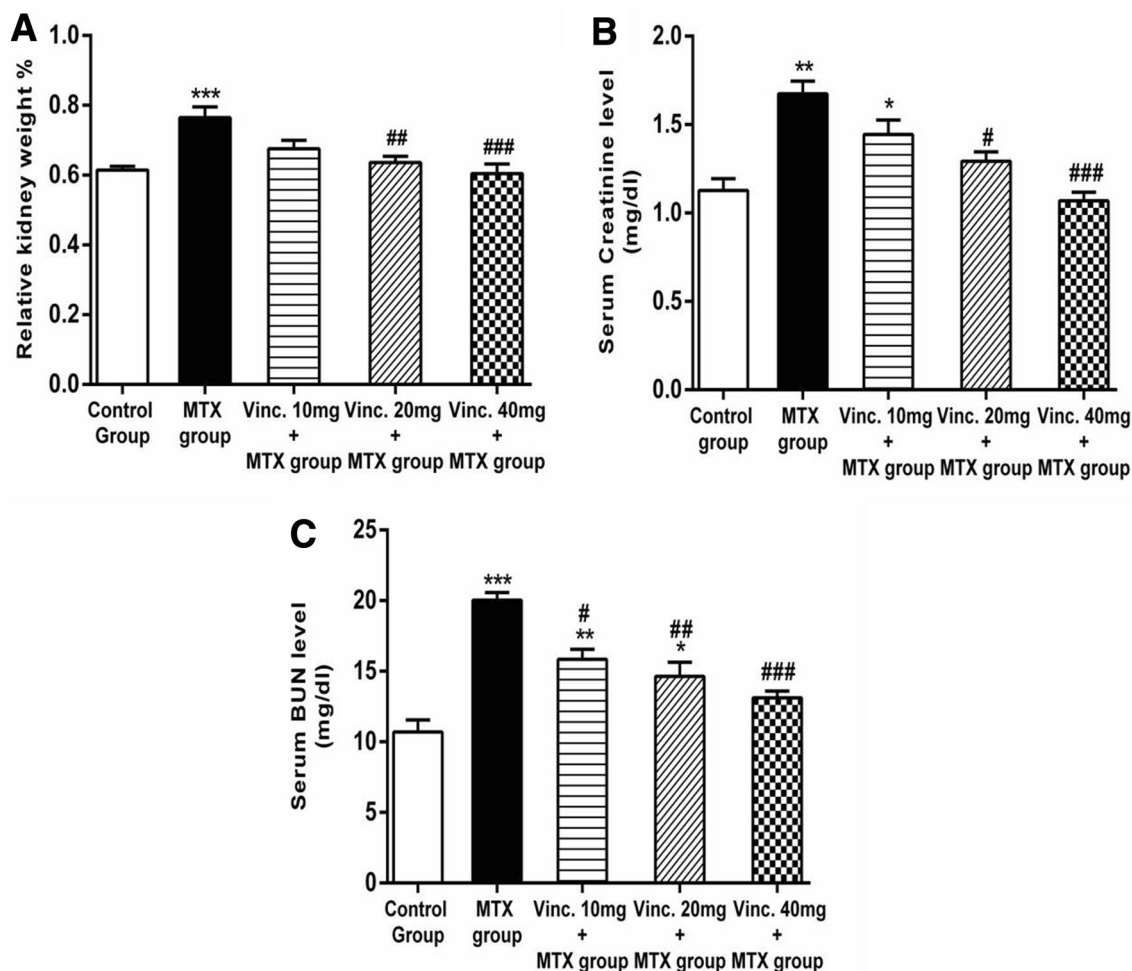
### Histopathological examination of kidney tissues

Examination of H and E-stained kidney sections revealed that the control group (Fig. 2a) showed no histopathological alteration. However, the systemic administration of MTX (20 mg/kg) caused a periglomerular focal hemorrhage (H) as well as inflammatory cell (M) infiltration that was detected in the periglomerular tissue. Also, degeneration was detected in the tubular lining epithelium associated with a vacuolization (V) in the lining endothelium of the glomerular tufts (Fig. 2b, c). Focal fibroblastic cells and collagen fiber (F) proliferation were detected in between the tubules at the cortex (Fig. 2d). There was congestion in the glomerular tufts as well as in the cortical blood vessels (Fig. 2e). There was focal hemorrhage (H) in between the degenerated tubules at the cortex as well as the corticomedullary portions (Fig. 2f). However, concomitant treatment with vincamine (10 mg/kg, p.o) showed focal inflammatory cell (M) infiltration in between the degenerated tubules at the cortex (Fig. 2g). Moreover, concomitant treatment with vincamine (20 mg/kg, p.o) showed congestion in the glomerular tufts (G) as well as the cortical blood vessels (B.V.) (Fig. 2h). However, concomitant treatment with vincamine (40 mg/kg, p.o) significantly attenuated MTX effect and relatively restored kidney histological features with only mild congestion in the glomerular tufts (Fig. 2i) and the corticomedullary junction showed normal intact histological structure of the tubules (Fig. 2j).

### Assessment of the mechanisms underlying vincamine protection against MTX-induced toxicity

#### Effects of vincamine on locomotor activity and Y-maze percent of alternation of MTX-treated rats

One-way ANOVA showed significant differences among groups on the percent change in locomotor activity and Y-maze percent of alternation test [ $F(3, 18) = 101.2$ ,  $P < 0.0001$  and  $F(3, 18) = 8.72$ ,  $P \leq 0.001$ , respectively] (Fig. 3a, b). Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant reduction in locomotor activity and Y-maze percent of alternation by 55.87% and 28.24%, respectively, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) for 10 days restored locomotor activity as they caused a significant increase in locomotor activity and Y-maze percent of alternation by 88.66% and 62.21%, as compared to MTX group and attenuated the effect of MTX



**Fig. 1** Effects of vincamine on relative kidney weight % (a), serum Cr. (b) and BUN level (c) of MTX-treated rats. Vincamine (10, 20, 40 mg/kg) was orally administered for 10 successive days in three different groups, respectively, and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, blood samples were collected, then animals were killed and kidneys were rapidly isolated and weighed in grams. Statistical analysis was

carried out by one-way ANOVA followed by Tukey multiple comparison test. Data are presented as mean  $\pm$  S.E.M ( $n=3$ ). \*Significantly different from control group at  $P<0.05$ . \*\*Significantly different from control group at  $P<0.01$ . \*\*\*Significantly different from control group at  $P<0.001$ . #Significantly different from MTX group at  $P<0.05$ . ##Significantly different from MTX group at  $P<0.01$ . ###Significantly different from MTX group at  $P<0.001$

in the brain tissues. Moreover, vincamine-alone-treated group did not show any significant changes in locomotor activity and Y-maze percent of alternation compared to the control group.

#### Effects of vincamine on step-through passive avoidance paradigm of MTX-treated rats

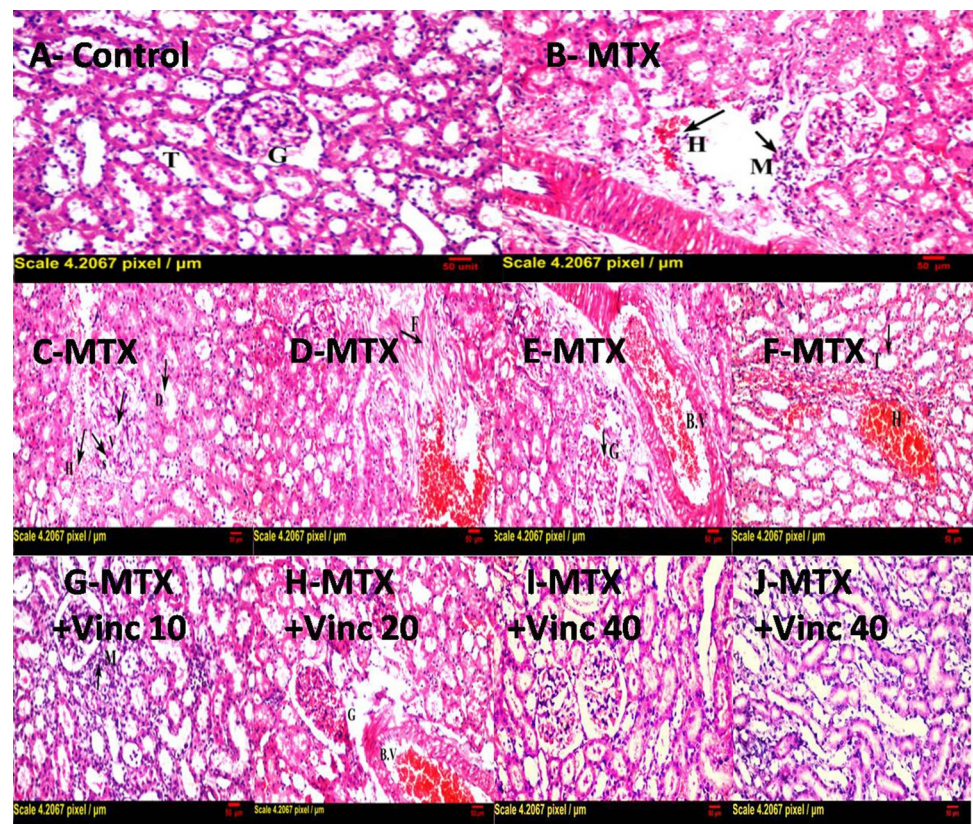
Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant decrease in retention latency [ $H(3, 18)=10.13, P=0.0175$ ] (Fig. 3c). Further statistical analysis with Dunn's test showed that MTX (20 mg/kg) resulted in a shorter latency to step-through compared to control groups, while concomitant treatment with vincamine (40 mg/kg) significantly attenuated MTX-induced amnesia.

Moreover, vincamine-alone-treated group did not show any significant changes in step-through latency compared to the control group.

#### Histopathological examination of brain tissues

Examination of H and E-stained kidney sections showed that systemic administration of MTX (20 mg/kg) caused nuclear pyknosis and degeneration in the neurons of both cerebral cortex and fascia dentata and hilus in the hippocampus. Also, focal hemorrhage along with multiple focal plague formation and diffuse gliosis were detected in the striatum. However, concomitant treatment with vincamine (40 mg/kg) did not induce as much of the MTX effect in the neurons of both cerebral cortex and fascia dentata in

**Fig. 2** H and E-stained sections of the MTX- and vincamine-treated rats. **a** Normal control kidney, **b–f** MTX-treated rats, **g** MTX- and vincamine (10 mg/kg)-treated group, **h** MTX- and vincamine (20 mg/kg)-treated group and **i, j** MTX- and vincamine (40 mg/kg)-treated group. Periglomerular inflammatory cell infiltration [M], focal hemorrhage [H], vacuolization [V], degeneration [D], focal fibroblastic cells proliferation [F], congestion of the glomerular tufts [G] and cortical blood vessels [B.V]



the hippocampus, but relatively restored brain histological features in the striatum with only mild vacuolization. However, control and vincamine-treated groups showed no histopathological alteration. Histopathological examination of the effect of different treatment groups on different brain areas is shown in Fig. 4a–o.

### Determination of oxidative stress biomarkers

#### Effects of vincamine on renal MDA, CAT and SOD levels of MTX-treated rats

One-way ANOVA showed significant differences among groups on the kidney MDA levels, CAT and SOD activities [ $F(3, 18) = 83.93$ ,  $F(3, 18) = 49.83$  and  $F(3, 18) = 29.95$ , respectively,  $P < 0.0001$ ] as shown in Table 1. Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant increase in kidney MDA level by 644.54%, but a significant decrease in kidney CAT and SOD activities by 46.06% and 71.48%, respectively, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg, p.o) for 10 days caused a significant decrease in kidney MDA level by 64.84%, but a significant increase in kidney CAT and SOD activities by 67.86% and 136.67% respectively, as compared to the MTX group attenuating the effect of MTX in the renal tissues. Moreover, vincamine-alone-treated

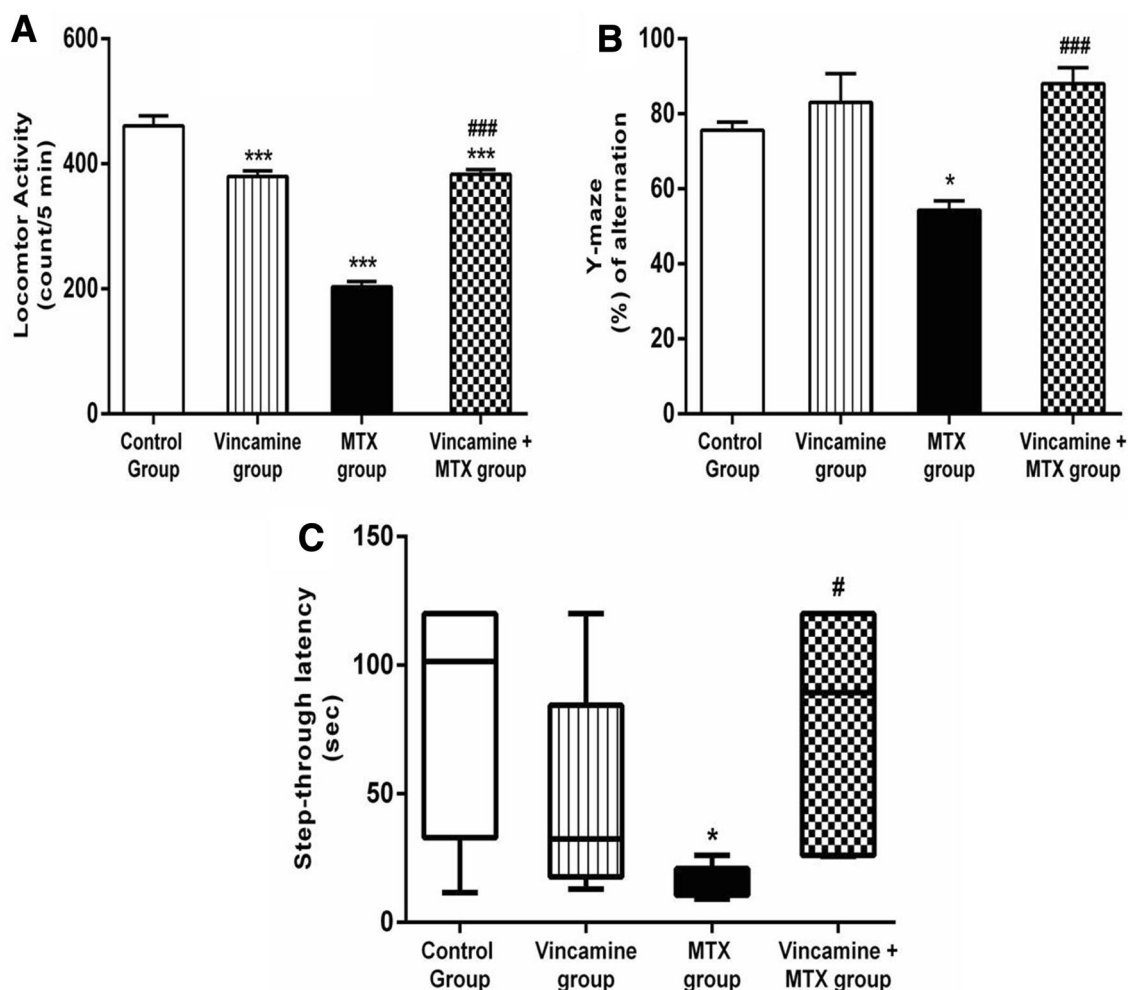
group did not cause any significant changes in kidney MDA levels, CAT and SOD activities compared to the control group.

#### Effects of vincamine on kidney Nrf2 and Ho-1 protein expression of MTX-treated rats

One-way ANOVA showed significant differences among groups on the percent change in kidney Nrf2 and HO-1 protein expression [ $F(3, 18) = 165.1$  and  $F(3, 18) = 78.37$ , respectively,  $P < 0.0001$ ] (Fig. 5a, b). Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant decrease in kidney Nrf2 and HO-1 protein expression by 78.67% and 79.33%, respectively, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) caused a significant increase in kidney Nrf2 and HO-1 protein expression by 265.68% and 299.95%, respectively, as compared to the MTX group. Vincamine-alone-treated group did not show any significant changes in kidney Nrf2 and HO-1 protein expression compared to the control group.

#### Effects of vincamine on kidney Nrf2 and Ho-1 mRNA expression of MTX-treated rats

One-way ANOVA showed significant differences among groups on the percent change in kidney Nrf2 and HO-1



**Fig. 3** Effects of vincamine on locomotor activity (a) and Y-maze % of alternation (b) of MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, locomotor activity assay and Y-maze alternation test were performed. Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparisons test. Data are presented as mean  $\pm$  SEM ( $n=6$ ). \*Significantly different from control group at  $P<0.05$ . \*\*\*Significantly different from control group at  $P<0.001$ . ###Significantly different from MTX group at  $P<0.001$ .

c Effects of vincamine on step-through passive avoidance paradigm of MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On days 9 and 10, training and test were performed, respectively. Statistical analysis was carried out by Kruskal–Wallis test followed by Dunn’s multiple comparison test. Data are presented as medians (25th, 75th percentile) ( $n=6$ ). \*Significantly different from control group at  $P<0.05$ . #Significantly different from MTX group at  $P<0.05$

mRNA expression [ $F(3, 18)=50.59$  and  $F(3, 18)=57.29$ , respectively,  $P<0.0001$ ] (Fig. 5c, d). Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant decrease in kidney Nrf2 and HO-1 mRNA expression by 80.07% and 86.28%, respectively, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) caused a significant increase in kidney Nrf2 and HO-1 mRNA expression by 324% and 502.26%, respectively, as compared to the MTX group. Vincamine-alone-treated group did not show any significant changes in kidney Nrf2 and HO-1 mRNA expression compared to the control group.

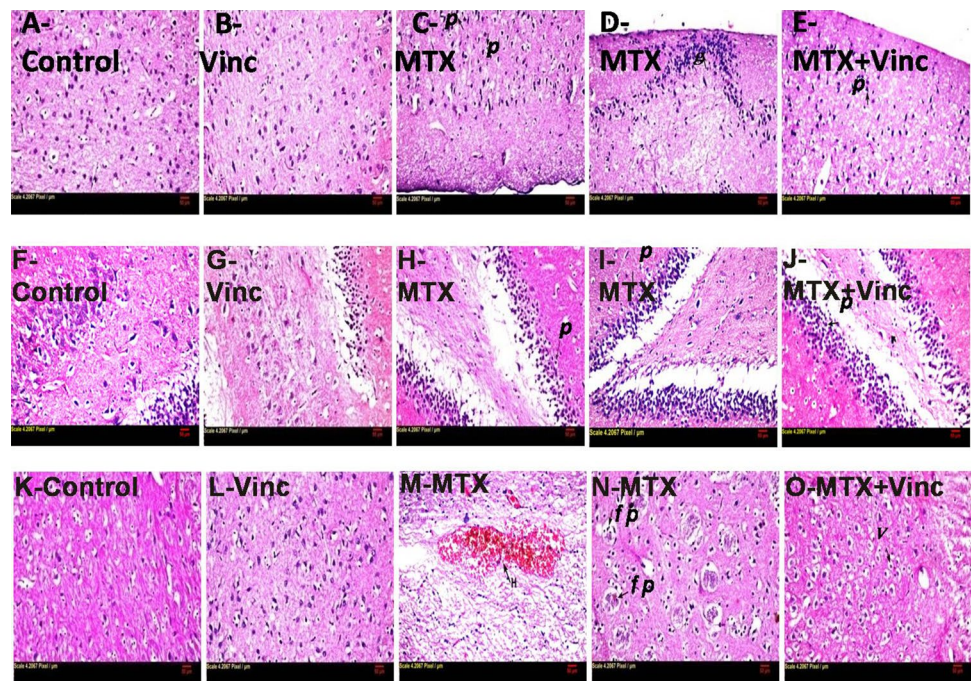
## Evaluation of Inflammatory markers

### Effects of vincamine on kidney NF- $\kappa$ B, IL-1 $\beta$ , IL-10, TNF- $\alpha$ and MPO levels of MTX-treated rats

One-way ANOVA showed significant differences among groups on the kidney NF- $\kappa$ B, IL-1 $\beta$ , IL-10, TNF- $\alpha$  and MPO levels [ $F(3, 18)=156.9$ ,  $F(3, 18)=145.6$ ,  $F(3, 18)=37.46$ ,  $F(3, 18)=268.7$  and  $F(3, 18)=56.13$ , respectively,  $P<0.0001$ ] (Fig. 6a–e). Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed



**Fig. 4** H and E staining of brain cerebral cortex (a–e), fascia dentata and hilus in the hippocampus (f–j) and striatum (k–o) of rats. a, f, k Normal control brain, b, g, l vincamine-only-treated rats, c, d, h, i, m, n MTX-treated rats and e, j, o: vincamine (40 mg/kg)- and MTX-treated rats. Nuclear pyknosis [p], focal gliosis [g], focal hemorrhage [H], multiple focal plaques [fp], vacuolization [V]



**Table 1** Effects of vincamine on kidney MDA, CAT, SOD levels of MTX-treated rats

Group	Parameter		
	MDA (mmol/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)
Normal control	8.5 ± 0.71	121.5 ± 2.47	3.8 ± 0.31
Vincamine (40 mg/kg)	8 <sup>###</sup> ± 0.67	130.7 <sup>###</sup> ± 2.77	4.3 <sup>###</sup> ± 0.39
MTX (20 mg/kg)	63.1 <sup>***</sup> ± 5.32	65.5 <sup>***</sup> ± 5.48	1.1 <sup>***</sup> ± 0.07
MTX (20 mg/kg) + vincamine (40 mg/kg)	22.2 <sup>*.###</sup> ± 1.69	110 <sup>###</sup> ± 4.81	2.6 <sup>*.###</sup> ± 0.14

Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, animals were killed and kidneys were rapidly isolated. Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparisons test. Data are presented as mean ± SEM ( $n=6$ )

\*Significantly different from control group at  $P < 0.05$

\*\*\*Significantly different from control group at  $P < 0.001$

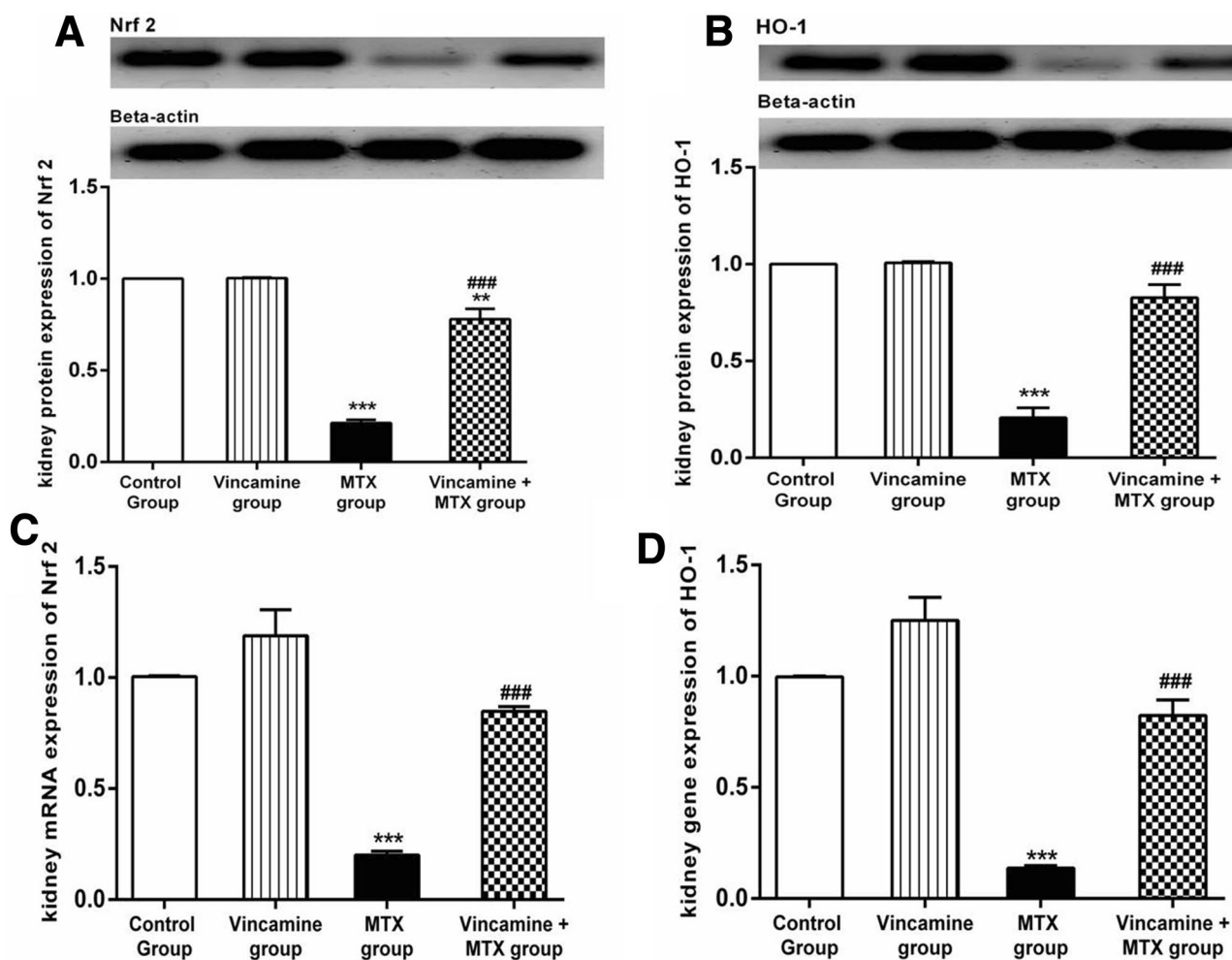
###Significantly different from MTX group at  $P < 0.01$

####Significantly different from MTX group at  $P < 0.001$

a significant increase in kidney NF- $\kappa$ B, IL-1 $\beta$ , TNF- $\alpha$  and MPO levels by 467.3%, 529.77%, 361.56% and 148.55%, respectively, while a significant decrease in IL-10 by 56.46% as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) for 10 days caused a significant decrease in kidney NF- $\kappa$ B, IL-1 $\beta$ , TNF- $\alpha$  and MPO levels by 55.57%, 54.59%, 56.32% and 49.89%, respectively, and significant increase in kidney IL-10 levels by 91.06%, as compared to MTX group. Moreover, vincamine-alone-treated group did not show any significant changes compared to the control group.

#### Effects of vincamine on renal COX-II expression of MTX-treated rats

Cox-II was assessed using immunohistochemical staining. Normal control group showed minimal immunohistochemical reactivity for Cox-II (Fig. 7a). Kidney sections from normal rats treated with vincamine alone did not show an elevation of Cox-II to a large extent and showed as faint brown staining (Fig. 7b). MTX treatment induced expression of Cox-II which was evident from intense brown staining (Fig. 7c). Conversely, treatment of kidney injured rats with vincamine significantly reduced the expression of Cox-II



**Fig. 5** Effects of vincamine on kidney Nrf2 (a) and Ho-1 (b) protein expression and mRNA expression (c, d), respectively, in MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, animals were killed and kid-

neys were rapidly isolated. Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparisons test. Data are presented as mean  $\pm$  SEM ( $n=6$ ). \*\*Significantly different from control group at  $P<0.01$ . \*\*\*Significantly different from control group at  $P<0.001$ . ###Significantly different from MTX group at  $P<0.001$

compared to MTX-treated group (Fig. 7d). The immunohistochemical staining was quantified as optical density (OD) of the stained regions (Fig. 7e) and area percent (A %) of expression of COX-II (Fig. 7f).

#### Western blot analysis of kidney caspase-3 protein expression in MTX-treated rats

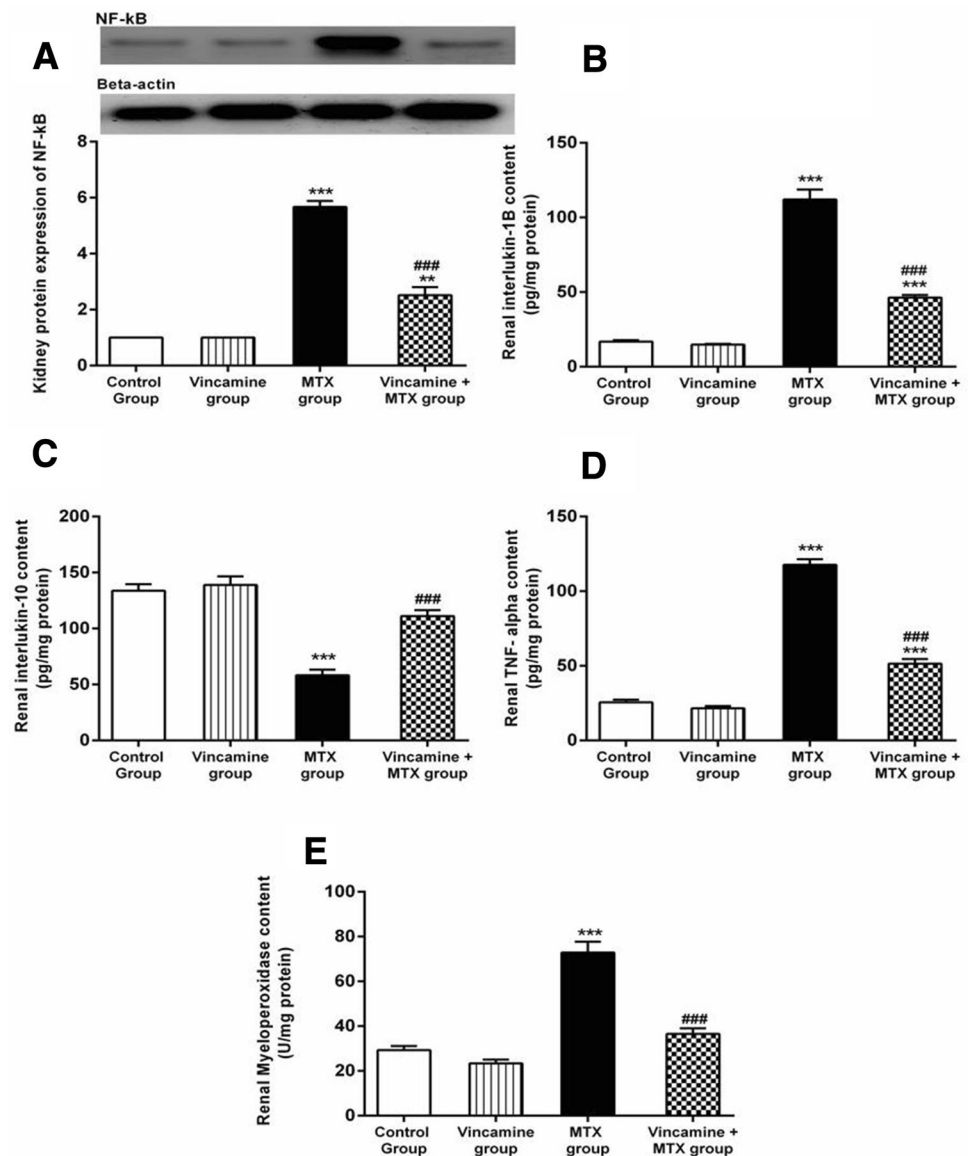
One-way ANOVA showed significant differences among groups on the percent change in kidney caspase-3 expression [ $F(3, 18)=105.8$ ,  $P<0.0001$ ] (Fig. 8a). Rats treated with MTX (20 mg/kg) showed significant increase in kidney caspase-3 level by 835.49%, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) days significantly decreased kidney caspase-3 level by 64.83% as compared to the MTX group.

Vincamine-alone did not cause any significant changes in kidney caspase-3 level if compared to the control group.

#### Effects of vincamine on kidney ATP level of MTX-treated rats

One-way ANOVA showed significant differences among groups kidney ATP levels [ $F(3, 18)=14.68$ ,  $P<0.0001$ ] (Fig. 8b). Rats that received MTX (20 mg/kg) on the 6th day of the experiment showed a significant decrease in kidney ATP levels by 53.41%, as compared to that of the normal control group. Concomitant treatment with vincamine (40 mg/kg) caused significant increase in kidney ATP level by 83.17% compared to MTX group. Moreover, vincamine treated group did not cause any significant changes in kidney ATP level if compared to the control group.

**Fig. 6** Effects of vincamine on kidney NF- $\kappa$ B (a), IL-1 $\beta$  (b), IL-10 (c), TNF- $\alpha$  (d) and MPO (e) levels of MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, animals were killed and kidneys were rapidly isolated. Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparisons test. Data are presented as mean  $\pm$  SEM ( $n=6$ ). \*\*Significantly different from control group at  $P<0.01$ . \*\*\*Significantly different from control group at  $P<0.001$ . ###Significantly different from MTX group at  $P<0.001$



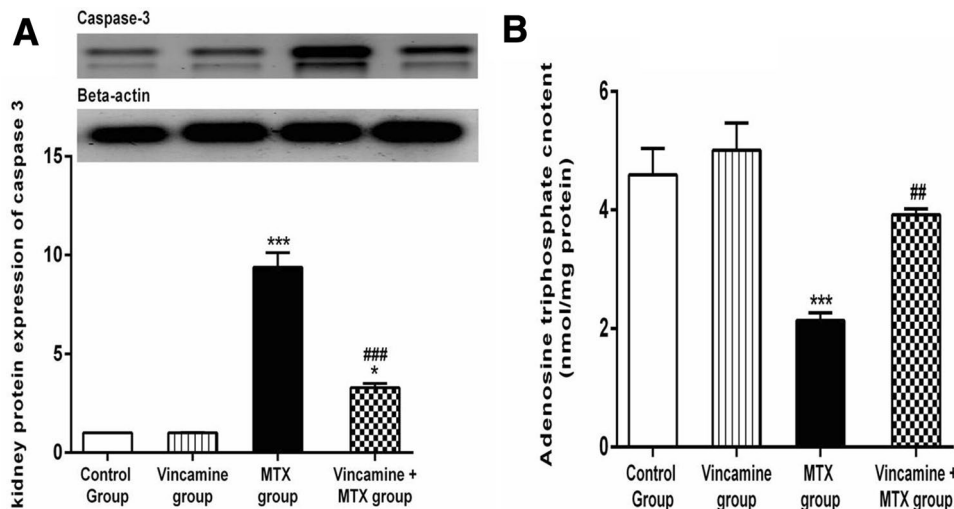
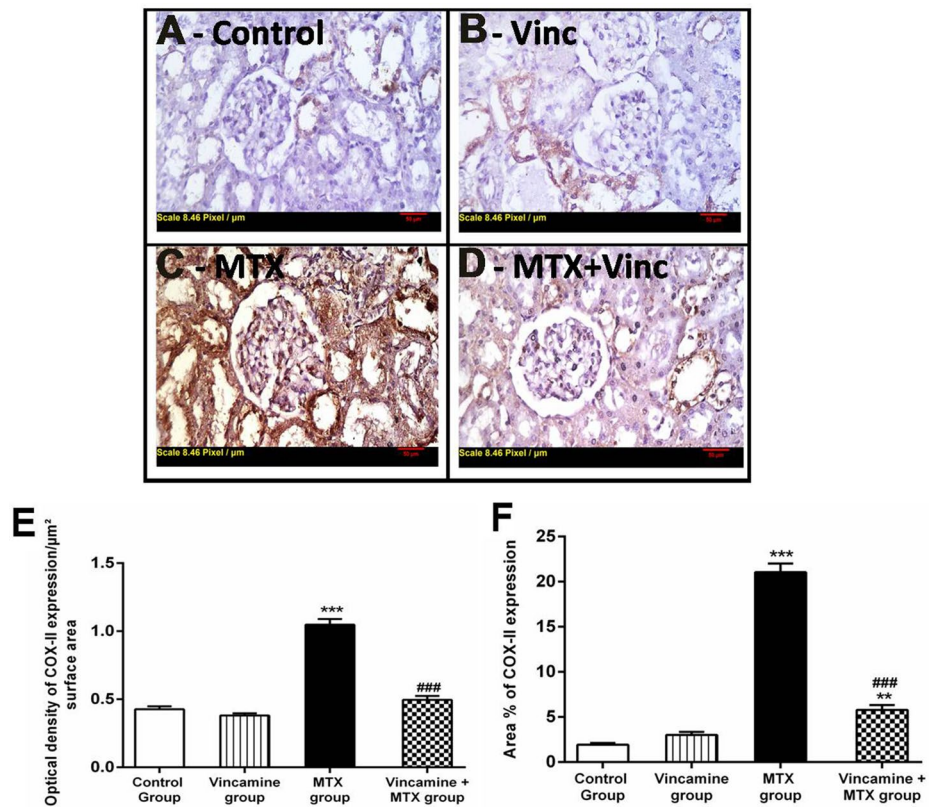
## Discussion

A number of protective measures were followed to reduce the incidence of HDMTX induced nephrotoxicity, which is considered as a life-threatening condition. However, renal dysfunction continues to occur in about 1.8% of patients with osteosarcoma treated on HDMTX clinical trials (Widemann and Adamson 2006). High mortality rate due to acute kidney injury (AKI) largely occurs due to extra-renal complications (Kelly 2006). AKI along with chronic kidney disease (CKD) is associated with neurological manifestations such as cognitive impairment, decreased consciousness and seizures. Those patients are indicated to start hemodialysis (Krishnan and Kiernan 2009; Liu et al. 2008). Uremic toxins accumulation is among the most common causes of uremic encephalopathy and cerebral

endothelial dysfunction in AKI and CKD (Bugnicourt et al. 2013). The present study aimed to investigate the protective effect of vincamine against MTX-induced kidney injury and the possible neuronal damages mediated through the kidney–brain axis.

The choice of vincamine was based on previous studies that demonstrated its ability to cross the blood–brain barrier and express a neuroprotective and antioxidant effects through the inactivation of hydroxyl free radicals. Moreover its derivative, vinpocetine, was found to exhibit neuroprotective effects in hippocampal neuronal cells (Abdel-Salam et al. 2016; Fayed 2010; Han et al. 2017; Zaki and Abdel-salam 2013). Vincamine has been also reported to protect against oxidative stress induced in liver (El-Dessouki et al. 2018). Its role in protecting against kidney injury, however, is still unexplored.

**Fig. 7** Effects of vincamine on renal COX-II expression of MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg, i.p) was administered on the 6th day of the experiment. On the last day of treatment, animals were killed and kidneys were rapidly isolated. Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparisons test. Data are presented as mean  $\pm$  SEM ( $n=6$ ). \*\*Significantly different from control group at  $P<0.01$ . \*\*\*Significantly different from control group at  $P<0.001$ . ###Significantly different from MTX group at  $P<0.001$



**Fig. 8** Western blot analysis of kidney caspase-3 protein expression (a) and evaluation of kidney ATP level (b) in MTX-treated rats. Vincamine (40 mg/kg) was orally administered for 10 successive days and MTX (20 mg/kg) was administered on the 6th day of the experiment. On the last day of treatment, animals were killed and kidneys were rapidly isolated. Statistical analysis was carried out by one-way

ANOVA followed by Tukey multiple comparisons test. Data are presented as mean  $\pm$  SEM ( $n=6$ ). \*Significantly different from control group at  $P<0.05$ . \*\*\*Significantly different from control group at  $P<0.001$ . ##Significantly different from MTX group at  $P<0.01$ . ###Significantly different from MTX group at  $P<0.001$

The results of the current study showed that MTX disrupted renal functions, manifested as an increase in serum Cr. and BUN levels that accumulate many other organic

anions derived from dietary proteins, which are considered as uremic toxins that cause uremic syndrome, metabolic defects and neurological symptoms (Deguchi et al. 2004;

Lisowska-Myjak 2014; Morsy et al. 2013). Uremic toxins were found to be a competitor with MTX for the organic-anion transporting polypeptides (OATPs) found in a variety of tissues of the body and considered important for MTX elimination, causing its disposition in renal tissues and resulting in further renal impairment as well as various tissues' negative effects (Lopez-Lopez et al. 2013; Nigam et al. 2015; Sato et al. 2014; VanWert and Sweet 2008).

The brain histopathological findings of the current study showed that MTX caused a nuclear pyknosis and degeneration in the cerebral cortex and in fascia dentata in hippocampus. Focal hemorrhage and vacuolization were noticed in most of the neurons of the striatum. These can explain the reduction in the locomotor activity and the deterioration of memory evidenced by the behavioral experiments. Concomitant vincamine treatment showed an improvement in the brain histopathological changes along with an enhancement in the locomotor activity, learning and memory functions.

Previous clinical and experimental studies have emphasized that any tissue exposed to an injurious event is perceived by macrophages and monocytes, which in turn secrete cytokines such as IL-1 and TNF- $\alpha$  (Asvadi et al. 2011). As evidenced in our present study, changes in the levels of IL-1 $\beta$ , IL-10 and TNF- $\alpha$  were consistent with the complex inflammatory response to MTX-induced kidney damage. TNF- $\alpha$  stimulates the activation of other inflammatory mediators including NF- $\kappa$ B, leading to expression of inflammatory genes such as inducible nitric oxide synthase along with COX-II which increases the synthesis of prostaglandins and leads to the perpetuation of inflammation (Shishodia 2013). As hypothesized in previous studies, one of the causes of MTX-induced hepatorenal damage is by induction of oxidative stress mediated by oxygen free radicals, which results in activating TNF- $\alpha$ , NF- $\kappa$ B, and COX-II inflammatory pathway. Such inflammatory process stimulates apoptotic events via increasing the expression of caspase-3 (Asvadi et al. 2011; El-Sheikh et al. 2015).

In agreement with the perception that the activation of the Nrf2/HO-1 pathway was coupled with antioxidant effects and was linked to the decreased levels of NF- $\kappa$ B and iNOS expression in experimental rats, as Nrf2 is important for the expression of antioxidant genes like SOD, CAT and HO-1 (Abd El-Twab et al. 2016; Mukherjee et al. 2013), we postulated that the improved efficacy of antioxidant nephroprotective defense may be explained by the ability of vincamine to regulate the Nrf2/HO-1 signaling pathway positively. Moreover, vincamine significantly depressed the TNF- $\alpha$  response and the inflammatory consequences; these were evidenced by our histopathological findings and the decreased level of COX-II immunohistochemical expression. In various models of renal diseases, it was stated that lipid peroxidation mediated by ROS has been suggested as a cause of cell death.

In addition to their direct damaging effects on tissues, ROS indirectly cause tissue injury through activation of neutrophils producing various enzymes such as MPO and proteases and release oxygen radicals in the tissue involved (Otuncemur et al. 2014). MTX-induced rise in MPO activity in the renal tissues was decreased by vincamine, suggesting that the antioxidant action of vincamine may be neutrophil dependent. Also, we assessed the elevation of MDA levels caused by MTX as an index for lipid peroxidation-induced kidney damage. MDA and MPO elevation together with a decrease in antioxidant enzymes SOD and CAT in renal tissues may suggest the direct toxicity mechanism of MTX through the oxidative stress and inflammatory consequences occurring in the kidneys. This was consistent with the findings of other studies (Asvadi et al. 2011; Hafez et al. 2015; Vardi et al. 2013). On the other hand, vincamine had decreased MDA and increased the antioxidants SOD and CAT levels; consequently, it decreased the renal tissue damage through the reduction of oxidative stress and enhancing the antioxidant defense mechanism in the kidneys.

ROS induced by oxidative stress cause an increase in mitochondrial electron transport and therefore a depletion in cellular ATP, resulting in Arabidopsis cell death (Tiwari et al. 2002). MTX had been reported to reduce the intracellular ATP levels of rat hepatocytes (Al Maruf et al. 2018; Walker et al. 2000). Vincamine has been previously reported to enhance cerebral metabolic processes through its influence on ATP production and efficient use of oxygen and glucose, while simultaneously providing increased safety against ischemia and hypoxia (Fayed 2010). In agreement with our findings, MTX significantly decreased renal ATP levels, while concomitant treatment with vincamine attenuated this suppression.

## Conclusion

In conclusion, vincamine reduced kidney injury and the consequent neurological damages, as it showed enhancement in behavioral activities and memory retention, and restored the histological abnormalities caused by MTX treatment. These activities could be, at least partially, explained based on its antioxidant, anti-inflammatory and the regulation of NF- $\kappa$ B, Nrf2/Ho-1 pathways, and that may be a promising target for the prevention or treatment of kidney injury. Further studies are needed to explain the exact mechanism of the protective effect of vincamine on MTX-induced cognitive impairment.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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