

## A DIHYDROCHALCONE AND SGLT2 INHIBITOR ABATE METHOTREXATE HEPATOTOXICITY IN MALE RATS; A COMPARATIVE STUDY

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### ABSTRACT

Drug-induced liver damage is a frequent adverse effect and a putative contributor to the acute liver damage associated with methotrexate (MTX) use. Both sodium/glucose cotransporter (SGLT2) inhibitor, empagliflozin (EMPA) and dihydrochalcone flavonoid, neohesperidin (NHD) have promising standards for abrogating oxidative stress, apoptosis, and inflammation. In the current study, we suggested that NHD and EMPA could show protecting properties against MTX-prompted liver harm while considering N-acetylcysteine (NAC) as a reference agent. To investigate our hypothesis, an experimental adult male rat model comprising of 70 rats divided randomly into 7 groups, was implemented. The impacts of MTX (20 mg/kg, once, i.p.), alone or with orally administered NHD (40 and 80 mg/kg), EMPA (10 and 30 mg/kg), and NAC (150 mg/kg) were assessed and paralleled to control group that received vehicle. Pretreatment with NHD and EMPA demonstrated marked enhancement in liver function aberrations, histopathological deterioration, and hepatic oxidative stress injuries, along with considerable downregulation of inflammatory cytokines TNF- $\alpha$  and IL-6. NHD and EMPA also showed notable decreases in NF- $\kappa$ B, Keap-1, caspase-3, and HSP70 expression levels, and an increase of Nrf2, PPAR $\gamma$ , and O-1 expressions. This investigation shows that both NHD and EMPA can mitigate oxidative injuries, apoptosis, and inflammation in hepatic tissues of MTX-remedied animals, primarily by the beginning of Nrf2/PPAR $\gamma$ /HO-1 signaling and suppression of NF- $\kappa$ B/IL-6/HSP70/TNF- $\alpha$  pathways. Both drugs represent a unique class that alleviates or at minimum impedes the starting of toxicity elicited by MTX; and may act as a promising therapeutic target for clinical use in the future.

**Keywords:** Methotrexate; empagliflozin; neohesperidin dihydrochalcone.

## Introduction

Drug-initiated hepatotoxicity is a recurrent adverse impact and a presumed contributor to acute liver damage (Khalifa et al. 2017). Methotrexate (MTX) chemotherapeutic drug is still deliberated a broadcaster drug for many diseases comprising, autoimmune disorders, cancer, and psoriasis (Ali et al. 2014). methotrexate (MTX) is widely used in cancer chemotherapy, with documented activity against leukemia, breast cancer, head and neck cancer, lymphoma, osteosarcoma, genitourinary cancer, and placental carcinoma (Erdogan et al. 2015). MTX interferes with dihydrofolate synthase, a crucial enzyme incorporated in tetrahydrofolate synthesis, whose inhibition leads to constraining of RNA and DNA synthesis by inhibition nucleotide production, thus exacerbating cell injury (Khalifa et al. 2017). In general, MTX swivels the cell to an extremely content of reactive oxygen species (ROS), which leads to mitochondrial abnormality, neutrophil infiltration and increased of NADPH oxidase (NOX) transcription (Mahmoud et al. 2017), and augmented expression amount of NOX (Arab et al. 2018; Erdogan et al. 2015). Excessive generation of ROS could persuade turbulences in the oxidative/antioxidant balance; and could speedy numerous redox-sensitive paths modification (Germoush et al. 2018), for instance initiation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), nuclear factor erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), Kelch ECH associating protein 1 (Keap1), and caspase-3, Heat shock proteins (HSPs) (Kelleni et al. 2016; De Maio. 1999).

Empagliflozin (EMPA) is a synthetic sodium/glucose co-transporter (SGLT) type II inhibitor, recently permitted in type II diabetes remedy, through enhancement renal elimination of glucose by constraining its reabsorption (Zhou et al. 2018). EMPA has been well known to inhibit mitochondrial deterioration and as a robust and consistent antioxidant potential profile (Abbas et al. 2018). EMPA limits the cell's progression to apoptosis through various antifibrotic mechanisms (Richter et al. 2018).

Neohesperidin dihydrochalcone (NHD) pertains to natural bicyclic flavonoids, which is deliberated as one of the substantial components of citrus fruits (Choi et al. 2007). NHD abolishes ROS triggered-cellular injury (Choi et al. 2007) and relieves the progress of apoptosis and inflammation (Wang and Cui. 2013; Graziano et al. 2012).

N-acetylcysteine (NAC) is eminent for its competence for oxidative and inflammatory mediators' abrogation and as a donor for the sulfhydryl group; subsequently, directing the cystine to production of glutathione (GSH) (Kahraman et al. 2013). NAC is well known, as oxidative insults removal, over and above its share in apoptosis regression (Kahraman et al. 2013); also, NAC incorporated in multiple conditions therapies, involving liver impairment, malignancies, and heart complications (Balansky et al. 2009; Khoshbaten et al. 2010).

This experimental work was conducted to inspect the putative protective actions of NHD and EMPA in abrogating of MTX-triggered hepatic injury.

This current study is a revised version of a previously published article (Osman et al. 2021) to correct the scientific record.

## Materials & methods

### Animals

In the current investigation, all experimental procedures and animal handling ethics aligned with the Ethics Committee of the Faculty of Medicine, Assiut University, Egypt (Permission No. 17200556). Eight-week-old male adult Swiss albino rats (weighing 190–220 g) were obtained from the Faculty of Medicine, Assiut University, Egypt. Rats were arbitrarily distributed into 14 cages (n=5) with free availability to diet and water and adapted to accommodate the conditions ( $28 \pm 2^\circ\text{C}$ ), humidity ( $50 \pm 15\%$ ) for 2 weeks before the starting the experiment.

### Drugs and chemicals

Methotrexate was bought from T3A (Assiut, Egypt). Empagliflozin was obtained from Boehringer Ingelheim (Germany), neohesperidin dihydrochalcone was bought from Sigma-Aldrich Chemical Company (USA), and N-acetylcysteine was obtained from SEDICO (6th October, Giza, Egypt. All drugs were suspended in 1 % carboxymethylcellulose (CMC) (Osman et al. 2021). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and total bilirubin (T. bilirubin) assay kits were provided by Bio-diagnostic Company (Cairo, Egypt). Lactate dehydrogenase (LDH) was obtained from Bio-system (Barcelona, Spain). Tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6) assay kits, immunohistochemical staining selected antibodies were purchased from Elabscience Biotech Company (USA).

### Experimental study

Seventy male adult Swiss albino rats were allocated into seven groups, with 10 animals in each group (n=10) as follows:

**Group I (Control):** animals received vehicle (1% CMC, p.o.) for 14 consecutive days.

**Group II (MTX control):** animals received vehicle (1% CMC, p.o.) for 14 consecutive days; injected with (20 mg/kg, once, i.p.) (Mahmoud et al. 2017) of MTX, towards the end of the seventh day from the start of the trial.

**Group III (MTX + EMPA10):** animals administered EMPA (10 mg/kg, p.o.) (Oelze et al. 2014) for 14 consecutive days; injected with (20 mg/kg, once, i.p. ) of MTX, towards the end of the seventh day from the start of the trial.

**Group IV (MTX + EMPA30):** animals were administered EMPA (30 mg/kg, p.o.) (Oelze et al. 2014) for 14 consecutive days; injected with (20 mg/kg, once, i.p. ) of MTX, towards the end of the seventh day from the start of the trial.

**Group V (MTX + NHD40):** animals received NHD (40 mg/kg, p.o.) (Wang and Cui. 2013) for 14 consecutive days; injected with (20 mg/kg, once, i.p. ) of MTX, towards the end of the seventh day from the start of the trial.

**Group VI (MTX + NHD80):** animals received NHD (80 mg/kg, p.o.) (Wang and Cui. 2013) for 14 consecutive days; injected with (20 mg/kg, once, i.p. ) of MTX, towards the end of the seventh day from the start of the trial.

**Group VII (MTX + NAC):** animals were given NAC (150 mg/kg, p.o.) (Khalifa et al. 2017) for 14 consecutive days; injected with (20 mg/kg, once, i.p. ) of MTX, towards the end of the seventh day from the start of the trial.

### **Serum and tissue preparation**

In the current trial, rats were prepared for surgical abdominal incision preceded by whole anesthesia; blood samples were withdrawn through left ventricle puncture. Sera samples were divided and promptly kept at  $-40\text{ }^{\circ}\text{C}$ . Instantly after the rat was euthanized, the liver of each animal was divided into three portions. The first portion was homogenized in twenty volumes in cold phosphate-buffered saline (PBS) and centrifuged at 3000 gx for a third of an hour at  $4\text{ }^{\circ}\text{C}$ . The lysate was harvested, separated into equal portions, and kept at  $-80\text{ }^{\circ}\text{C}$  for biochemical assessment. The other portion was instantly immersed in a 10 % buffered formalin solution in normal saline for additional histopathological examination. After that the last portion was instantly dipped into liquid nitrogen and retained at  $-80\text{ }^{\circ}\text{C}$  for molecular analyses.

### **Evaluation of hepatic function parameters**

By commercially obtainable kits, ALT and AST were investigated calorimetrically by Reitman and Frankel method (Reitman and Frankel. 1957). LDH was evaluated as lined by Izquierdo and Dias method (Izquierdo and Dias. 1983). Finally, levels of albumin and T. bilirubin were measured as the method of Lowry (Lowry et al. 1951) and Martinen (Martinen. 1966) were lined, respectively.

### **Evaluation of pro-inflammatory mediators**

Pro-Inflammatory cytokines TNF- $\alpha$  and IL-6 of the hepatic tissue were assessed by quantitative ELISA kits as stated by the 'manufacturer's protocol (Van Weemen and Schuurs. 1971).

### **Evaluation of oxidative stress parameters**

Liver contents of GSH, TBARS, NO<sub>2</sub><sup>-</sup>, and TAC were evaluated calorimetrically consistent with the principles defined previously by Ellman (Ellman. 1959), Uchiyama and Mihara (Uchiyama and Mihara. 1978), Montgomery and Dymock (Montgomery and Dymock. 1961), and Koracevic et al. (Koracevic et al. 2001), respectively. The antioxidant enzymes: GST and SOD levels were assessed as stated by Keen et al. (Keen et al. 1976) and Marklund (Marklund. 1985), respectively.

### **Histopathological investigation**

Liver tissue pieces were divided and pinned in 10% neutral buffered formalin for 3 days, then treated with serial grades of ethanol, cleared in xylene, infiltrated with paraplast tissue inserting media and then fixed out into tissue blocks. Tissues were cut into pieces (4 µm thickness) *via* a rotary microtome and attached on glass slides. Followed by staining with hematoxylin and eosin as a common examination staining method for appearing histological abnormalities (Bancroft and Gamble. 2008).

### **Hepatic genes expression investigation**

Total hepatic RNA was extracted using Sepal reagent (Nacalai Tesque, Inc., Kyoto, Japan), and then the first-strand cDNA was made using Rever-Tra Ace (Code No. FSK-101100, Toyobo Co., Ltd., Osaka, Japan) and using random primer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After that, PCR was conducted using StepOnePlus Real-Time PCR with Fast SYBR Green Master Mix Reagent (Cat: 4309155, Thermo Fisher Scientific, Inc.) and the selected primers (Table 1). The relative gene expression was determined using the comparison CT technique and normalized to the β-actin gene as an endogenous reference gene as mentioned in Livak and Schmittgen method (Livak and Schmittgen. 2001).

**Table 1:** Sequencing of Qrt-PCR primers

Gene	Primer sequence (5'-3')
Caspase-3	F: GGTATTGAGACAGACAGTGG R: CATGGGATCTGTTTCTTTGC
Keap1	F: TCAGCTAGAGGCGTACTGGA R: TTCGGTTACCATCCTGCGAG
PPAR $\gamma$	F: GGACGCTGAAGAAGAGACCTG R: CCGGGTCCTGTCTGAGTATG
HO-1	F: GTAAATGCAGTGTTGGCCCC R: ATGTGCCAGGCATCTCCTTC
NF- $\kappa$ B	F: TGGGACGACACCTCTACACA R: GGAGCTCATCTCATAGTTGTCC
Nrf2	F: TTGTAGATGACCATGAGTCGC R: TGTCCCTGCTGTATGCTGCTT
$\beta$ -actin	F: GGGAAATCGTGCGTGACATT R: GCGGCAGTGGCCATCTC

(Keap1, kelch-like ECH-associated protein 1; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma, HO-1, heme oxygenase-1; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; and Nrf2, nuclear factor erythroid 2-related factor 2).

### Immunohistochemical study

Paraffin-embedded tissue sections (4  $\mu$ m thick) were obtained from different animal groups. Immunohistochemical staining was done as stated by manufacturer's protocols. Deparaffinized tissue slices were treated by 3 % H<sub>2</sub>O<sub>2</sub> for twenty minutes, followed by incubation with polyclonal antibodies (rabbit) against HSP70 Santa Cruz Biotechnology Inc., (1:50) and HO-1 (Elabscience - E-AB - 10366 {1:50) at 4°C overnight; washing, followed by secondary antibody HRP Envision kit (DAKO) incubation for twenty minutes; washing by PBS and incubated with diaminobenzidine for fifteen minutes. Washing by PBS then counterstaining with hematoxylin, dehydration, and clearing in xylene, then coverslipped. 6 random non-overlapping fields per sample were chosen at random from each section for determination of area % of immunoexpression levels of HO-1 and HSP70 in hepatic tissue samples. All micrographs and morphological measurements were analyzed, and photographs were obtained by the Leica Application module for histological analysis attached to a full HD microscopic imaging system (Leica Microsystems GmbH, Germany).

### Western blotting

Equal amounts (25  $\mu$ g) of protein samples were mixed and boiled with SDS loading buffer for 5 minutes, allowed to cool on ice for 7 minutes and then loaded into SDS-polyacrylamide gel and separated by Cleaver electrophoresis unit (Cleaver, UK), transferred onto polyvinylidene fluoride (DF) membranes (BioRad) for half an hour using a Semi-dry Electrobloetter (Biorad, USA) at 2.5 A and 25 V for half an hour. The

membrane was treated with 5 % nonfat dry milk in TBS-T for two hours at 37° C, to diminish non-specific protein interactions; incubated at 4° C overnight with each primary antibody at specified dilution (PPAR $\gamma$  (1:500), Nrf2 (1:500), and  $\beta$ -actin (1:1000). Then allowing Nrf2, PPAR- $\gamma$ , and  $\beta$ -actin primary antibodies to react against their corresponding proteins. The resultant blots were now washed (3 times, 10 minutes each) with TBS-T. The corresponding horseradish peroxidase linked secondary antibodies (Dako) were then incubated with the membrane for 1 hour at room temperature; followed by washing (3 times, 10 minutes each) with TBS-T. The blot was then, treated with chemiluminescent Western ECL substrate (Perkin Elmer, Waltham, MA) as stated by the manufacturer's protocol. The chemiluminescent signals were taken using a Chemi Doc imager (Biorad, USA); bands intensity was measured and normalized to  $\beta$ -actin.

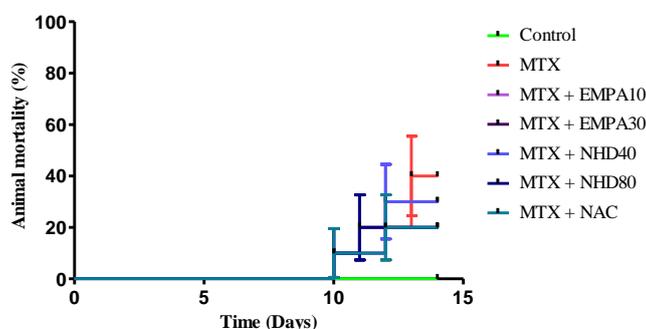
### Statistical analysis

Data analyses are shown as a mean  $\pm$  standard error of the mean (SEM). Differences between mean values of normally distributed data were analyzed by a one-way analysis of variance (ANOVA) test followed by a Tukey-Kramer test for post hoc analysis. All measurements were implemented 3 times independently to lessen standard error as possible. Statistical significance was accepted at a level of  $p < 0.05$ . The survivability curve was made *via* Kaplan- Meier manner by GraphPad Prism version 7.0 (GraphPad software San Diego, USA).

## Results

### Effects of NHD and EMPA on survivability rates of MTX rats

The animal mortality rates for each group were recorded daily over the course of 2 succeeding weeks of the experiment. Then Kaplan-Meier survivability analysis was executed, and a curve was extracted. MTX injection increased the death percentage to 40% at the last day of the experiment. In contrast, pretreatment of NHD and EMPA for 2 succeeding weeks showed a decrease in death rates to 30%, 20%, 30%, 20%, and 20%, for EMPA10, EMPA30, NHD40, NHD80, and NAC groups, respectively, at the same time (Fig. 1). Notably, the differences regarding mortality rates for the NHD and EMPA groups were negligible.



**Fig. 1:** Impacts of NHD and EMPA on mortality rates of MTX rats.

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine).

### NHD and EMPA ameliorate hepatic impairment of MTX rats

In the existing study, ALT, LDH, AST, and T. bilirubin levels were noticeably increased in MTX-remedied rats (Table 2). Contrariwise, pretreatment with NHD and EMPA for 2 succeeding weeks produced substantial reductions in ALT, LDH, AST, and T. bilirubin levels paralleled to MTX-remedied group, respectively (Table 2). Of note, pretreatment with NHD and EMPA did not show remarkable differences in the ALT, LDH, AST, and T. bilirubin levels.

**Table 2:** Effects of NHD and EMPA on liver biomarkers of MTX rats.

Parameter	Control	MTX	MTX+ EMPA10	MTX+ EMPA30	MTX+ NHD40	MTX+ NHD80	MTX+ NAC
ALT (U/ml)	55 ± 2.5	230 ± 5 <sup>a</sup>	190 ± 12 <sup>a,b</sup>	145 ± 6 <sup>a,b</sup>	170 ± 16 <sup>a,b</sup>	130 ± 12 <sup>a,b</sup>	150 ± 5 <sup>a,b</sup>
AST (U/ml)	52 ± 4	217 ± 9 <sup>a</sup>	130 ± 6 <sup>a,b</sup>	129 ± 4 <sup>a,b</sup>	138 ± 4 <sup>a,b</sup>	170 ± 12 <sup>b</sup>	180 ± 17 <sup>b</sup>
LDH (U/L)	1300 ± 54	4500 ± 195 <sup>a</sup>	3470 ± 192 <sup>a,b</sup>	3190 ± 182 <sup>a,b</sup>	3070 ± 200 <sup>a,b</sup>	3000 ± 282 <sup>a,b</sup>	3280 ± 147 <sup>a,b</sup>
T. bilirubin (mg/dl)	0.7 ± 0.01	1.8 ± 0.08 <sup>a</sup>	1.3 ± 0.09 <sup>a,b</sup>	1.2 ± 0.08 <sup>a,b</sup>	1.1 ± 0.07 <sup>a,b</sup>	1 ± 0.06 <sup>b</sup>	1.1 ± 0.09 <sup>a,b</sup>

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; T. bilirubin, total bilirubin).

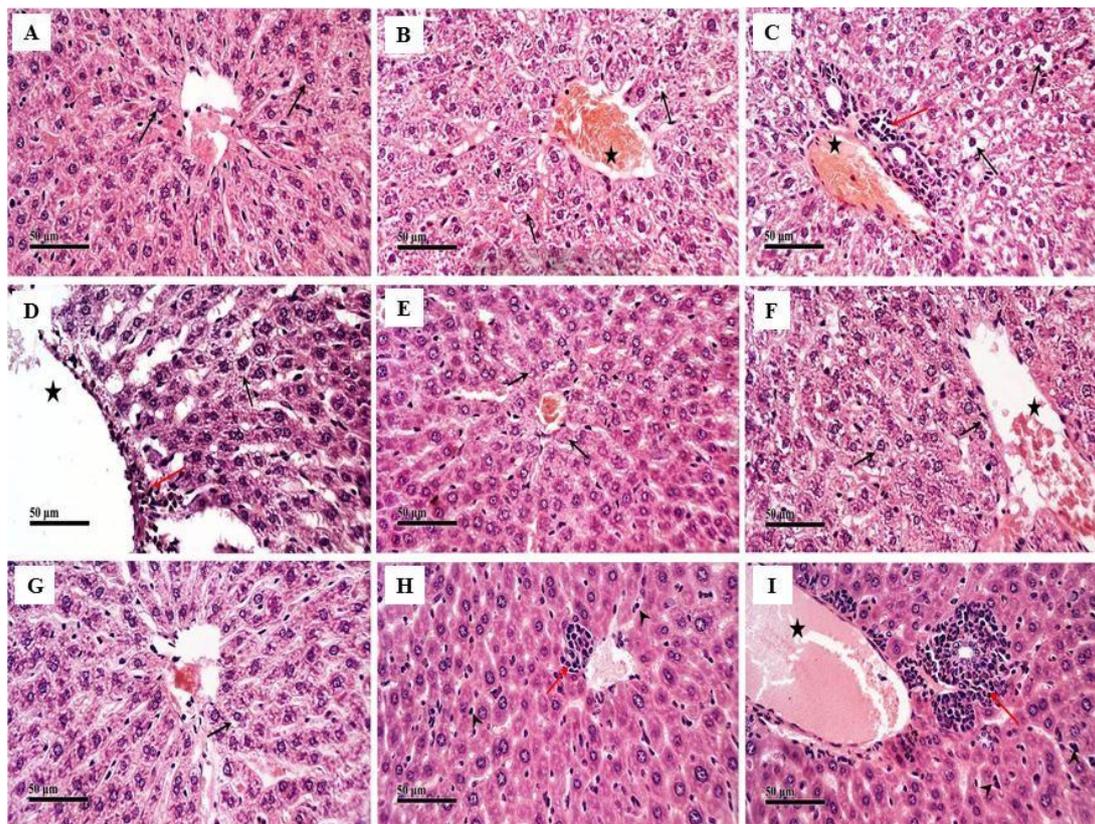
Statistical data are showed as means ± SEM, *n* = 6.

<sup>a</sup>*p* < 0.05 paralleled to control group.

<sup>b</sup>*p* < 0.05 paralleled to MTX group.

### NHD and EMPA prevent histopathological abrasions prompted by MTX

Microscopic investigation of different liver tissue section from MTX-injected group; exhibited degenerative vacuolation of the hepatocyte in most of the hepatic sections with numerous pyknotic nuclei, with moderate vasculitis (Fig. 2 (B, C)). Control group section showed normal histological structures of hepatocyte with several obvious integral hepatocytes and cellular structures (arrow) and normal hepatic vasculature (Fig. 2 (A)). Conversely, pretreatment with EMPA10, EMPA30, NHD40, NHD80, and NAC for 2 succeeding weeks showed an improvement in hepatocellular degeneration, congestion, vasodilation, and inflammatory cell infiltration paralleled to the sections of MTX-provoked group (Fig. 2 (D-I)), respectively. Conspicuously, hepatic tissues of NHD and EMPA groups did not display marked morphological alterations between each other.



**Fig. 2:** Effects of NHD and EMPA on histopathological damage in MTX-triggered hepatic injury. Constituent photomicrographs of liver segments stained with (H & E X 400). (A) control group, (B & C) MTX group reveals diffuse hepatocellular vacuolar degeneration (arrow), moderate vascular dilation and congestion (star), and inflammatory cells infiltrate (red arrow), (D) EMPA10-treated group, (E) EMPA30-treated group, (F) NHD40-treated group, (G) MTX + NHD80, and (H & I) NAC-treated group show higher records of activated Kupffer cells (arrowhead).

(H & E; hematoxylin and eosin, NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine).

### **NHD and EMPA lessen liver inflammation of MTX rats**

The IL-6 and TNF- $\alpha$  pro-inflammatory mediators' levels were noticeably raised versus control rats (Table 3). Quite the reverse, NHD and EMPA administration for 2 succeeding weeks triggered marked declines in IL-6 and TNF- $\alpha$  expression paralleled to MTX-injected rats. Remarkably, NHD and EMPA pretreatment display insignificant differences in TNF- $\alpha$  and IL-6 levels.

**Table 3:** Effects of NHD and EMPA on hepatic IL-6 and TNF- $\alpha$  inflammatory mediators of MTX rats.

Parameter	Control	MTX	MTX + EMPA10	MTX + EMPA30	MTX+ NHD40	MTX + NHD80	MTX+ NAC
IL-6 (ng/mg protein)	76 $\pm$ 3	400 $\pm$ 12 <sup>a</sup>	190 $\pm$ 12 <sup>a,b</sup>	180 $\pm$ 12 <sup>a,b</sup>	180 $\pm$ 15 <sup>a,b</sup>	170 $\pm$ 12 <sup>a,b</sup>	180 $\pm$ 3.5 <sup>a,b</sup>
TNF- $\alpha$ (ng/mg protein)	210 $\pm$ 7	670 $\pm$ 15 <sup>a</sup>	370 $\pm$ 12 <sup>a,b</sup>	290 $\pm$ 19 <sup>a,b</sup>	350 $\pm$ 12 <sup>a,b</sup>	260 $\pm$ 12 <sup>b</sup>	280 $\pm$ 17 <sup>a,b</sup>

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; IL-6, Interleukin-6; TNF- $\alpha$ , Tumor necrosis factor-alpha).

Statistical data are showed as means  $\pm$  SEM,  $n = 6$ .

<sup>a</sup> $p < 0.05$  paralleled to control group.

<sup>b</sup> $p < 0.05$  paralleled to MTX group.

### NHD and EMPA reduce hepatic oxidative stress of MTX rats

The hepatic level of GSH and TAC have prominently exhausted in MTX-administered rats, versus control rats (Table 4). Contrariwise, pretreatment with NHD and EMPA for 2 succeeding weeks revealed significant rises in GSH and TAC contents, paralleled to MTX-administered rats (Table 4). The hepatic level of TBARS and NO<sub>2</sub><sup>-</sup> were risen expressively in MTX-group paralleled to control one (Table 4). Quite the opposite, pre-treatment with NHD and EMPA for 2 succeeding weeks revealed significant reductions in TBARS and NO<sub>2</sub><sup>-</sup> contents, paralleled to MTX-treated animals (Table 4). The SOD and GST levels were notably declined in rats injected with MTX paralleled to control animals (Table 4). Conversely, administration of NHD and EMPA for 2 succeeding weeks showed notable elevations in GST activity and SOD level, versus MTX-injected rats (Table 4). Remarkably, noticeable alterations between NHD and EMPA groups were excluded in these previous parameters.

**Table 4:** Effects of NHD and EMPA on liver oxidative parameters of MTX animals.

Parameter	Control	MTX	MTX +EMPA10	MTX+EMPA30	MTX + NHD40	MTX + NHD80	MTX+ NAC
GSH( $\mu\text{mol/mg}$ protein)	4.9 $\pm$ 0.25	1.56 $\pm$ 0.05 <sup>a</sup>	2.8 $\pm$ 0.12 <sup>a,b</sup>	4.8 $\pm$ 0.3 <sup>b</sup>	3.1 $\pm$ 0.16 <sup>a,b</sup>	4.6 $\pm$ 0.4 <sup>b</sup>	4.55 $\pm$ 0.04 <sup>b</sup>
TBARS (nmol/mg protein)	15.4 $\pm$ 0.7	9 $\pm$ 1.5 <sup>a</sup>	25 $\pm$ 1.2 <sup>a,b</sup>	19 $\pm$ 1.3 <sup>b</sup>	23 $\pm$ 1.6 <sup>a,b</sup>	18 $\pm$ 1.1 <sup>b</sup>	18 $\pm$ 1.7 <sup>b</sup>
NO <sub>2</sub> <sup>-</sup> ( $\mu\text{mol/mg}$ protein)	14 $\pm$ 0.02	29 $\pm$ 0.1 <sup>a</sup>	20 $\pm$ 0.01 <sup>a,b</sup>	15 $\pm$ 0.12 <sup>a,b</sup>	19 $\pm$ 0.03 <sup>a,b</sup>	13 $\pm$ 0.10 <sup>a,b</sup>	16 $\pm$ 0.1 <sup>a,b</sup>
TAC (mmol/mg protein)	1.8 $\pm$ 0.02	0.61 $\pm$ 0.03 <sup>a</sup>	0.88 $\pm$ 0.06 <sup>a,b</sup>	0.93 $\pm$ 0.03 <sup>a,b</sup>	0.90 $\pm$ 0.04 <sup>a,b</sup>	0.98 $\pm$ 0.01 <sup>a,b</sup>	0.90 $\pm$ 0.05 <sup>ab</sup>
SOD (U/ $\mu\text{g}$ protein)	0.1 $\pm$ 0.002	0.05 $\pm$ 0.004 <sup>a</sup>	0.065 $\pm$ 0.002 <sup>a,b</sup>	0.080 $\pm$ 0.007 <sup>b</sup>	0.069 $\pm$ 0.0054 <sup>a,b</sup>	0.089 $\pm$ 0.006 <sup>b</sup>	0.075 $\pm$ 0.004 <sup>a,b</sup>
GST (U/ $\mu\text{g}$ protein)	3 $\pm$ 0.12	1.1 $\pm$ 0.001 <sup>a</sup>	1.3 $\pm$ 0.01 <sup>a,b</sup>	1.55 $\pm$ 0.10 <sup>a,b</sup>	1.1 $\pm$ 0.03 <sup>a,b</sup>	1.90 $\pm$ 0.18 <sup>a,b</sup>	1.9 $\pm$ 0.08 <sup>a,b</sup>

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; GSH, reduced glutathione; TBARS, thiobarbituric acid reactive substances; NO<sub>2</sub><sup>-</sup>, nitrite; TAC, total antioxidant capacity; SOD, superoxide dismutase; GST, glutathione S-transferase).

Statistical data are showed as mean  $\pm$ SEM,  $n = 6$ .

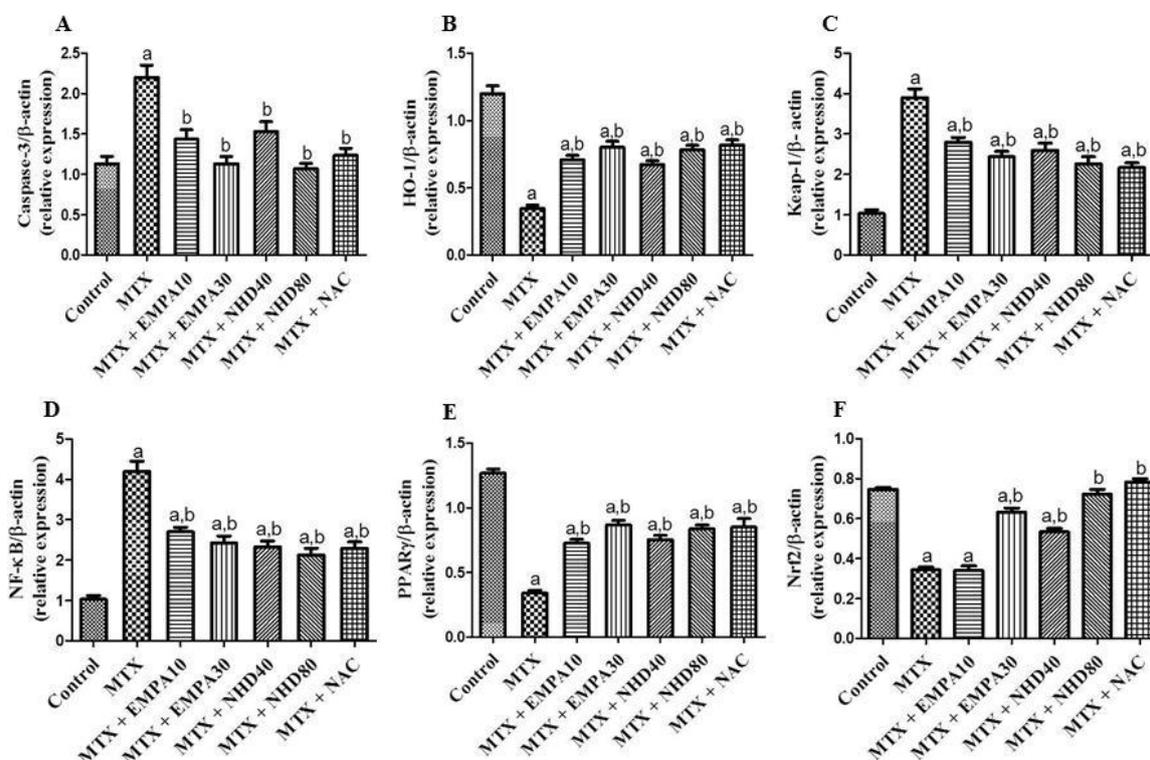
<sup>a</sup> $p < 0.05$  paralleled to control group.

<sup>b</sup> $p < 0.05$  paralleled to MTX group.

### Impacts of NHD and EMPA on hepatic genes expression levels of MTX rats

In contrast, to control rats, MTX injection resulted in significant rises in the gene expression of Keap1, caspase-3, and NF- $\kappa$ B (Fig. 3 (A, C, D)). In reverse, NHD and EMPA exposure for 2 succeeding weeks induced significant declines in caspase-3 and in Keap1, and in NF- $\kappa$ B expression levels, versus MTX rats (Fig. 3 (A, C, D)). The hepatic HO-1, PPAR $\gamma$ , and Nrf2mRNA expressed levels were noticeably reduced in MTX rats versus the normal rats (Fig. 3 (B, E, F)). Contrariwise, pretreatment with NHD and EMPA for 2 succeeding weeks induced obvious raises in HO-1, PPAR $\gamma$ , and in Nrf2 mRNA expression level, versus MTX-administered animals (Fig. 3 (B, E, F)). Outstandingly, NHD and EMPA

pretreatment shown insignificant differences between rats in the PPAR $\gamma$ , caspase-3, HO-1, Keap1, Nrf2, and NF- $\kappa$ B genes expression levels.



**Fig. 3:** Impacts of NHD and EMPA on caspase-3 (A), HO-1 (B), Keap1 (C), NF- $\kappa$ B (D), PPAR $\gamma$  (E), and Nrf2 mRNA (F) genes expression levels in MTX-induced liver injury.

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; Keap1, kelch-like ECH-associated protein 1; HO-1, heme oxygenase-1; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; NF- $\kappa$ B; nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2).

Statistical data are showed as mean  $\pm$ SEM,  $n = 3$ .

<sup>a</sup> $p < 0.05$  paralleled to control group.

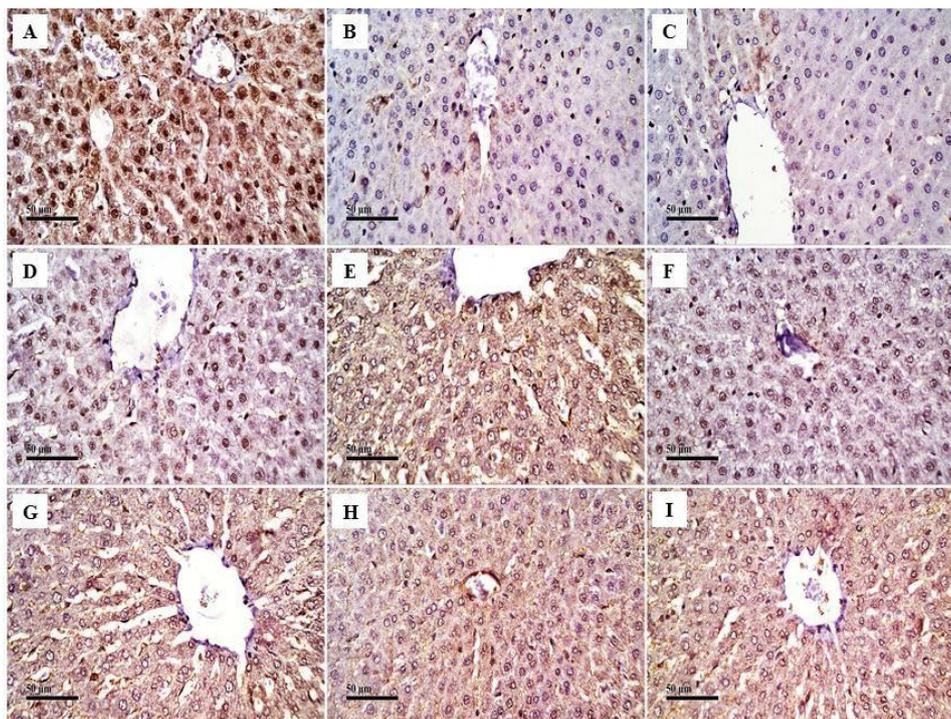
<sup>b</sup> $p < 0.05$  paralleled to MTX group.

### Effects of NHD and EMPA on HO-1 and HSP-70 proteins expression levels of MTX rats

In contrast with control animals, hepatic HO-1 protein expression level was significantly reduced in MTX rats (Fig. 4 & 6(A)). Alternatively, NHD and EMPA

pre-treatment for 2 succeeding weeks shown noticeable upregulation in HO-1 protein level, when paralleled to MTX-received rats (Fig. 4 & 6(A)).

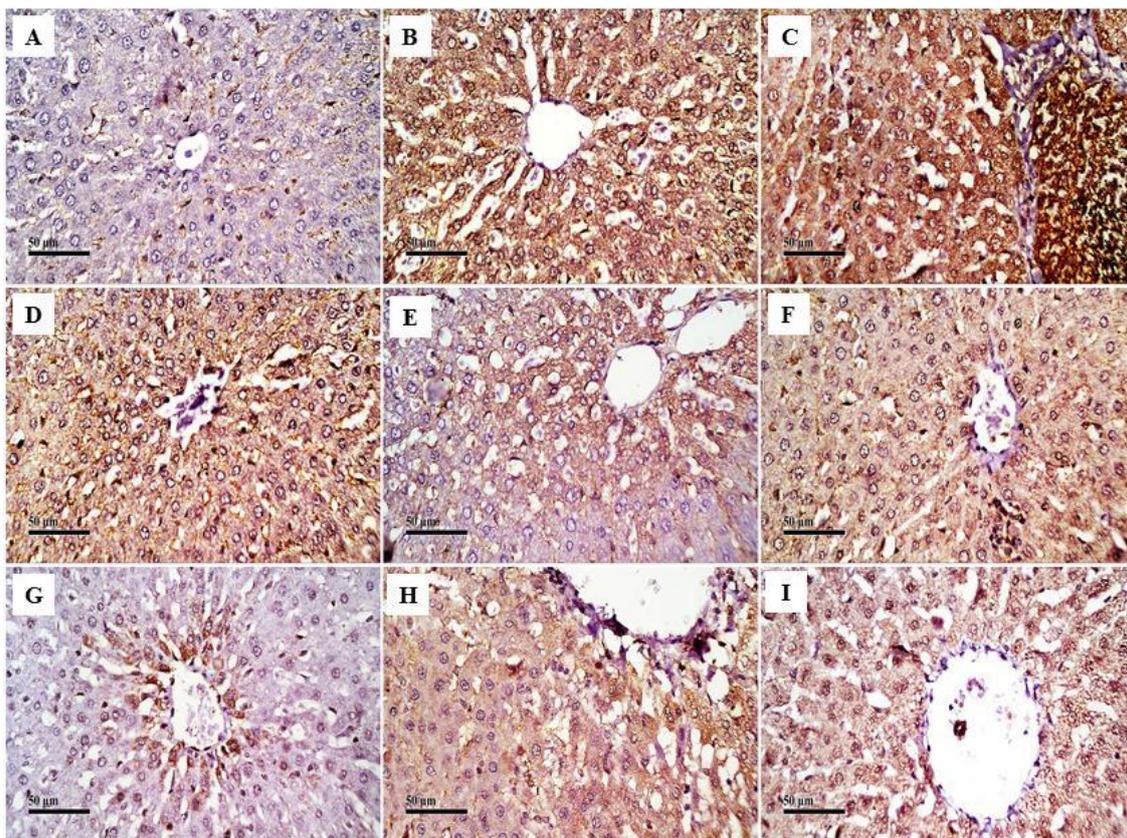
MTX exposure once produced a marked rise in the level of HSP70 expression (Fig. 5 & 6(B)). Otherwise, NHD and EMPA exposure for 2 succeeding weeks provoked a remarkable lessening in the level of HSP70 expression (Fig. 5 & 6(B)). In general, pretreatment with NHD and EMPA exhibited insignificant variances in the HSP70 and HO-1 expression levels at their groups.



**Fig. 4:** Effects of NHD and EMPA on HO-1 protein expression level in MTX-provoked liver injury.

Photo (A): Immunohistochemical HO-1 staining in rat liver from normal control sample shows predominant protein expression level. Photo (B, C): MTX samples reveal a large decrease in HO-1 expression level. Photo (D, E): EMPA10 and EMPA30 administration show an increase in HO-1 protein expression levels. Photo (F, G): Immunohistochemical examination of the effect of NHD40 and NHD80 administration on HO-1 protein expression level in MTX-induced liver injury in rats. Photo (H, I): Immunohistochemical examination of the effect of NAC administration on HO-1 protein expression level in MTX-induced liver injury in rats.

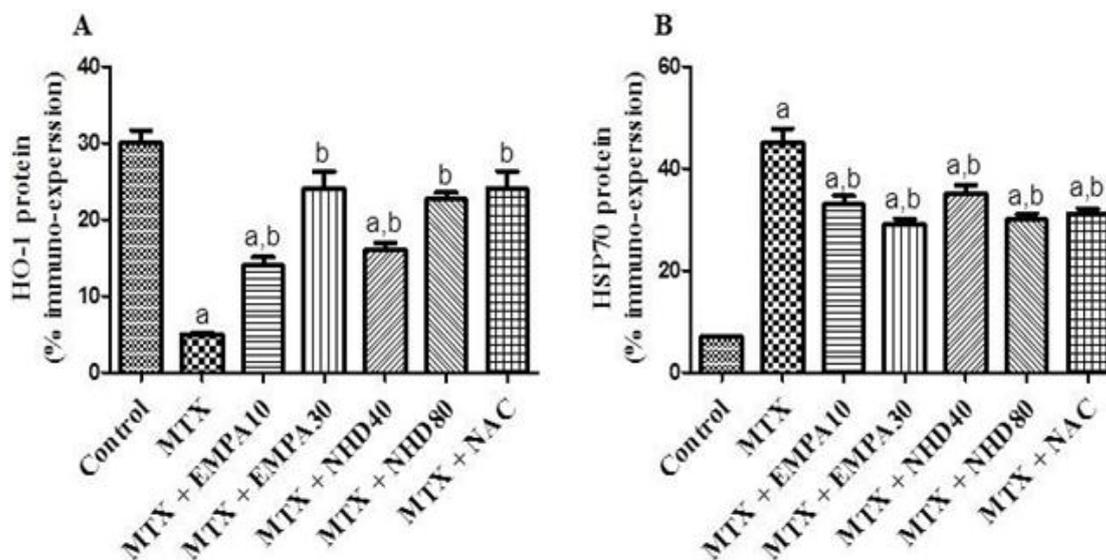
(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; HO-1, heme oxygenase-1).



**Fig. 5:** Effects of NHD and EMPA on hepatic HSP70 protein expression level in MTX-provoked liver toxicity.

Photo A: Immunohistochemical HSP70 staining in rat liver from normal control section, showing predominant protein expression level; Photo (B, C): MTX show an increase in HSP70 expression level. Photo (D, E): Immunohistochemical examination of the effect of EMPA10 and EMPA30 administration on HSP70 protein expression level in MTX-induced liver injury in rats. Photo (F, G): Immunohistochemical examination of the effect of NHD40 and NHD80 administration on HSP70 protein expression level in MTX-induced liver injury in rats. Photo (H, I): Immunohistochemical examination of the effect of NAC administration on HSP70 protein expression level in MTX-induced liver injury in rats.

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; HSP70, heat shock protein 70).



**Fig. 6:** Semi-quantitative immunoexpression analysis on the expression levels of HSP70 and HO-1 proteins.

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; HSP70, heat shock protein 70; HO-1, heme oxygenase-1).

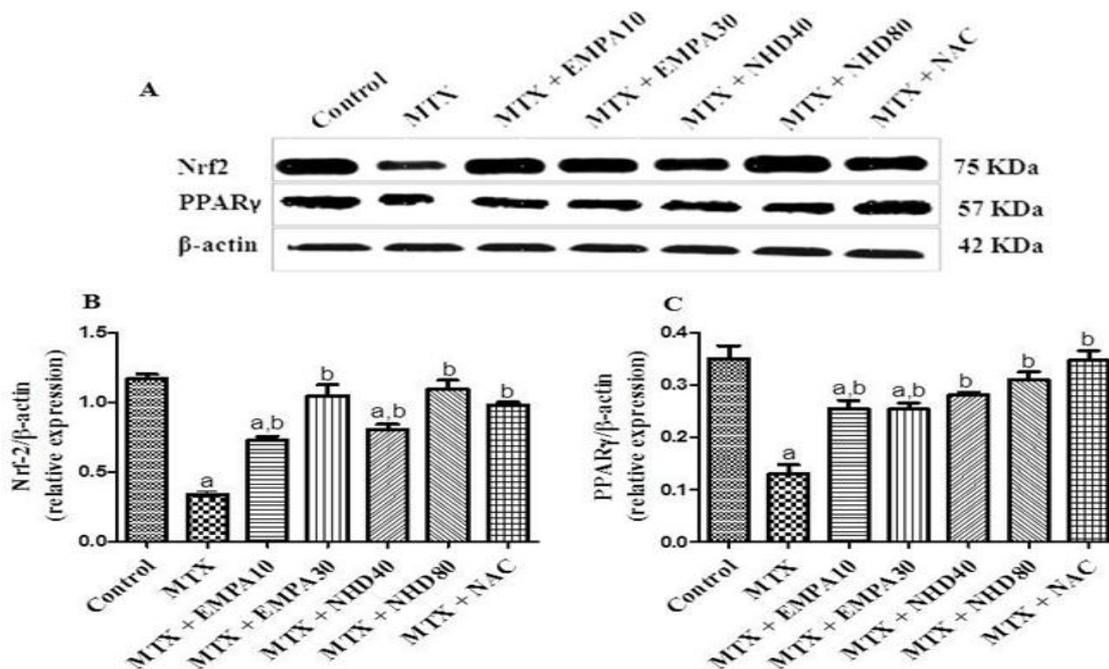
Statistical data are showed as mean  $\pm$ SEM, n = 3.

<sup>a</sup>p<0.05 paralleled to control group.

<sup>b</sup>p<0.05 paralleled to MTX group.

### **NHD and EMPA up-regulate Nrf2 and PPAR $\gamma$ expression levels in MTX-triggered liver damage**

We investigated the impacts of NHD and EMPA administration on the liver Nrf2 and PPAR $\gamma$  proteins expression levels. Paralleled with control animals, the expression of Nrf2 and PPAR $\gamma$  levels were prominently reduced in MTX-remedied rats (Fig. 7 (A&B&C)), respectively. Contrariwise, the pre-treatment with NHD and EMPA for 2 following weeks exhibited significant rises in Nrf2 and PPAR $\gamma$  expression level versus MTX-remedied group (Fig. 7 (A&B&C)), respectively. Remarkably, pre-treatment with NHD and EMPA showed no marked differences between them in the Nrf2 and PPAR $\gamma$  expression levels.



**Fig. 7:** Effects of NHD and EMPA on hepatic Nrf2 and PPAR $\gamma$  expressed proteins in MTX-incited liver detriment. Typical gel of Nrf2 and PPAR $\gamma$  proteins levels standardized to  $\beta$ -actin (A); statistical analysis of Nrf2 and PPAR $\gamma$  proteins levels (B & C), respectively.

(NHD, neohesperidin dihydrochalcone; MTX, methotrexate; EMPA, empagliflozin; NAC, N-acetylcysteine; Nrf2, nuclear factor erythroid 2-related factor 2; PPAR $\gamma$ , peroxisome proliferator-activated receptor).

Statistical data are showed as mean  $\pm$ SEM, n = 3.

<sup>a</sup>p<0.05 paralleled to control group.

<sup>b</sup>p<0.05 paralleled to MTX group.

## Discussion

MTX is classified as a disease-modifying antirheumatic drug that reduces inflammation and autoimmune diseases by suppressing the immune system. The therapeutic benefits of MTX are usually connected with its related severe toxicity (Mahmoud et al. 2014). It has been lined that MTX administration resulted in deterioration of liver function biomarkers and resultant liver injury via proliferation of oxidative, apoptotic, and inflammatory mediators (Erdogan et al. 2015). AST, ALT, LDH, T. bilirubin levels, and histopathological analysis are crucial pointers for evaluating hepatocellular integrity. Moreover; assessment of survival rates could reflect the degree of MTX toxicity acceptance (Khalifa et al. 2017). The results of the current study showed that MTX (20 mg/kg) injection, caused significant declines in survival rates, and rises in the level of AST, ALT, LDH, and T. bilirubin. Provoked hepatotoxicity triggered by MTX injection was established by

morphological modifications, which were proven by abundant hepatic pyknotic nuclei, and moderate vascular congestion with infiltration of the inflammatory cell. These demonstrations were in synchronization with formerly reported studies (Ali et al. 2014). Pretreatment with NHD and EMPA revealed marked decreases in ALT, AST, LDH, T. bilirubin levels, as well as death rates; and hence protected the hepatic architecture against the pathological alterations. Similarly, pre-treated with NHD and EMPA exhibited a noticeable improvement of normal liver morphology and its circulating biomarkers. These investigations came in accordance with previous studies (Shi et al. 2015; Lai et al. 2020).

We too inspected TNF- $\alpha$  and IL-6 tissue levels, two sensitive inflammatory mediators principally elaborated in valuing the inflammation (Magari et al. 2003). In our study, MTX injection provoked marked rises in TNF- $\alpha$  and IL-6 levels (Matata and Galiñanes. 2002) (Chandra and Sten. 2002; Mahmoud et al. 2014), although exposure to NHD and EMPA presented a remarkable decrease in TNF- $\alpha$  and IL-6 levels. Such results may be elucidated by alleviating NF- $\kappa$ B and redox-sensitive transcription factors levels and constraining peroxynitrite formation. These influences were in consonance with preceding articles (Shi et al. 2015; Ojima et al. 2015).

The oxidative/antioxidant inequity shows a vigorous role in evaluating the magnitude of tissue damage (Ali et al. 2014). In this respect, the assessment of the impacts of oxidative injuries-mediated damage can be assessed by quantifying TBARS, NO<sub>2</sub><sup>-</sup> TAC, and GSH contents. Owing to GST and SOD activities enzymes, which are sensitive parameters, they are primarily elaborated in assessing the grade of ROS damage (Lamlé et al. 2008). In our research MTX administration was notably related to decreased content of GSH and TAC and repressed activity of GST and SOD enzymes sideways with increase in the content of NO<sub>2</sub><sup>-</sup> and TBARS (Khalifa et al. 2017; Mahmoud et al. 2017). NHD and EMPA pretreatment showed a significant lessening of MTX-induced ROS in liver tissue. This can be linked to the decrease in NOX-4 expression level, instigation of cytoplasmic Nrf2 transportation to the nucleus, and inspiration of Nrf2/antioxidant response element expression levels; eventually leads to reticence of oxidative injuries liberation. These results were in harmony with preceding investigators who stated that NHD and EMPA mitigate oxidative stress in experimentally reported models (Larsen et al. 2017; Wu et al. 2017).

Here, to inspect the primary molecular paths of subsequent oxidative injury, apoptotic alterations, and inflammation, Nrf2/PPAR $\gamma$ /HO-1 and NF- $\kappa$ B/Keap1/HSP70/caspase-3 signaling paths were also involved. Under oxidative stress stimuli, Nrf2 shows a critical role after detachment from Keap1 to augment the cytoprotective agents' expression level (Lamlé et al. 2008); Keap1 manages Nrf2 liberation and inhibits its exhaustion from the cytoplasm (Kerr et al. 2017). PPAR $\gamma$  persuades the expression levels of antioxidant enzyme and disables NF- $\kappa$ B and NOX activity (Polikandriotis et al. 2005; Adcock et al. 2011). NF- $\kappa$ B is considered as one of the chief managers of immune-inflammatory reactions and is comprised in the cytokines production regulation (Germoush et al. 2018). Caspase-3 is a key pro apoptotic marker of cell viability

that is activated by both endogenous and exogenous cellular triggers (Yuluğ et al. 2019). Heat shock proteins (HSPs) are renowned initiated chaperones that are mostly conjugated with cell damage; ubiquitously over-expressed to lessen oxidative injury, and revamp cellular function (Kelleni et al. 2016). HSP-70 is amid the utmost concordant explored HSPs as a cytoprotective remedial target (De Maio. 1999). Furthermore, HO-1 theaters a significant role in degradation of cellular oxidative stress (Jiang et al. 2017). In the present investigation, MTX injection produced a noticeable reduction in PPAR $\gamma$ , Nrf2, and HO-1 expression at gene and protein level. Conversely, MTX injection presented a marked upturn in gene expression levels of NF- $\kappa$ B, caspase-3, Keap1, and HSP70 protein. These investigations were in harmony with many earlier results (Kamel et al. 2016; Mahmoud et al. 2017; Yuluğ et al. 2019). These molecular paths alterations were inverted by NHD and EMPA pretreatment. It might be linked with the protective actions of NHD and EMPA against oxidative stimuli and inflammation of hepatocytes. These effects are associated with enhanced PPAR $\gamma$ , Nrf2 and HO-1 expression levels and reduced NF- $\kappa$ B, Keap1, caspase-3, and HSP70 expression levels (Wang and Cui. 2013; Li et al. 2019).

## Conclusion

This investigation takes new perceptions into the presumed protective effects of both NHD and EMPA on liver damage elicited by MTX. NHD and EMPA guard against hepatotoxicity convinced by MTX, mostly by instigation of Nrf2/PPAR $\gamma$ /HO-1 signaling and suppression of NF- $\kappa$ B/IL-6/HSP70/TNF- $\alpha$  axis. This indicates that promising results could be achieved in clinical trials; NHD and EMPA would protect against MTX or at least delay its adverse effects.

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## يحد ثنائي هيدروكالكون ومثبط ناقل مشترك الصوديوم والجلوكوز-٢ من السمية الكبدية للميثوتريكسيت في ذكور الجرذان؛ دراسة مقارنة

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السمية الكبدية الناتجة عن تناول الأدوية هي تأثير ضار متكرر ومساهم مفترض في تلف الكبد الحاد المرتبط باستخدام الميثوتريكسيت. كل من مثبطات ناقل الصوديوم/الجلوكوز الإمباغليفلوزين والنيوهيسبيريدين لديهم معايير واعدة لإبطال الإجهاد التأكسدي، موت الخلايا المبرمج، والالتهاب. في الدراسة الحالية.

اقترحنا أن الإمباغليفلوزين و النيوهيسبيريدين يمكن أن يظهر تأثيرات وقائية ضد إصابة الكبد التي تسببها الميثوتريكسيت مع اعتبار الاستيل سيستين كمعيار مرجعي للتجربة.

من أجل التحقيق من فرضيتنا ، تم تنفيذ نموذج تجريبي من الجرذان الذكور يتكون من ٧٠ جرذاً بالغاً مقسماً بشكل عشوائي إلى سبع مجموعات: مجموعة الميثوتريكسيت (٢٠ مجم/كجم، تم حقنها مرة واحدة في الغشاء البريتوني)، بمفردها أو مع نيوهيسبيريدين المعطى عن طريق الفم (٤٠ و ٨٠ مجم/كجم/يوم)، إمباغليفلوزين (١٠ و ٣٠ مجم/كجم/يوم) والاستيل سيستين (١٥٠ مجم/كجم/يوم) تم تقييمهم وموازيتهم لمجموعة التحكم العادية التي تلقت المذيب فقط.

أظهرت المعالجة المسبقة بالإمباغليفلوزين و النيوهيسبيريدين تحسناً ملحوظاً في انحرافات وظائف الكبد ، والتدهور المرضي للانسج ، وإصابات الإجهاد التأكسدي الكبدية ، جنباً إلى جنب مع انخفاض التنظيم في مستوى الوسطاء الالتهابيين (انترلوكين-٦، عامل نخر الورم الفأ).

أظهر إمباغليفلوزين و نيوهيسبيريدين أيضاً انخفاضاً ملحوظاً في مستويات التعبير لكلا من كيش شبيه انزيم اينويل كوايه الماءى البروتين المرتبط ١ ، العامل المعزز لسلسلة كابا ٣-كاسباز و بروتين الصدمة الحرارية ٧٠ ، وزيادة في مستويات التعبير لـ العامل النووي المتعلق بالعامل-٢ ، مستقبل منشط بمكاثير البيروكسيسوم النوع جاما و هيم اوكسيجيناز-١. يوضح هذا التحقيق أن كلاً من الإمباغليفلوزين و نيوهيسبيريدين يمكن أن يخفف من الإجهاد التأكسدي ، والاستماتة ، والتكاثر الالتهابي في الأنسجة الكبدية للحيوانات المعالجة بالميثوتريكسيت، في المقام الأول عن طريق بداية إشارات (العامل النووي المتعلق بالعامل-٢ - مستقبل منشط بمكاثير البيروكسيسوم النوع جاما - هيم اوكسيجيناز-١) وقمع مسارات (العامل المعزز لسلسلة كابا- بروتين الصدمة الحرارية ٧٠- انترلوكين-٦ - عامل نخر الورم الفأ) الخلوية.

كلا من العقارين يمثلان فئة فريدة من نوعها قد تخفف أو تعيق على الأقل بدء السمية التي تثيرها الميثوتريكسيت ؛ وقد يكون بمثابة هدف علاجي واعد للتطبيق السريري في المستقبل.

**الكلمات المفتاحية:** الميثوتريكسيت، إمباغليفلوزين ونيوهيسبيريدين ثنائي هيدروكالكون.