

# Berberine mitigates methotrexate-induced oxidative stress and inflammation in the cerebrum of rats

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## ARTICLE INFO

### Article history:

Received on: 14/09/2017

Accepted on: 22/10/2017

Available online: 30/11/2017

### Key words:

Methotrexate, Inflammation, Berberine, Oxidative stress.

## ABSTRACT

Berberine is a natural isoquinoline alkaloid with multiple beneficial therapeutic effects. This study was designed to evaluate the protective effect of berberine against methotrexate (MTX)-induced oxidative stress and inflammation in the brain of rats. Rats received a single intraperitoneal injection of MTX (20 mg/kg) and orally administered 25 mg/kg and 50 mg/kg body weight berberine for 7 days. MTX-induced rats showed significantly increased lipid peroxidation and nitric oxide levels in the cerebrum. Treatment of the MTX-induced rats with berberine produced a significant decrease in cerebral levels of lipid peroxidation and nitric oxide. In addition, berberine induced a significant increase in reduced glutathione, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase in the cerebrum of MTX-induced rats. Rats received MTX showed a significant up-regulation of nuclear factor-kappaB (NF- $\kappa$ B), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1beta (IL-1 $\beta$ ) expression in the cerebrum, an effect that was significantly reversed following treatment with berberine. In conclusion, berberine protects against MTX-induced neurotoxicity through attenuating oxidative stress and inflammation, and boosting the antioxidant defenses.

## INTRODUCTION

The dihydrofolate reductase inhibitor methotrexate (MTX) is an anti-metabolite used frequently to treat autoimmune diseases and as a chemotherapeutic agent against many types of cancer (Ayad *et al.*, 2014, Sakthiswary and Suresh, 2014, Zhu *et al.*, 2014). In the treatment of rheumatoid arthritis and psoriasis, MTX represents the favored steroid-sparing immunosuppressant (Roeningk *et al.*, 1998). Acute, sub-acute, and long-term neurotoxicities of MTX have been well-established (Buizer *et al.*, 2006; Inaba *et al.*, 2008). Disruption of central nervous system (CNS) folate homeostasis and/or direct neuronal damage are the mechanisms through which MTX exerts neuro-toxicity (Cole

*et al.*, 2009; Vezmar *et al.*, 2009). Studies have demonstrated that subacute MTX neurotoxicity occurs 2 to 14 days after high-dose or prolonged low-dose oral MTX. This neurotoxicity manifests with seizures, aphasia, encephalopathy and stroke-like symptoms (Asato *et al.*, 1992; Rubnitz *et al.*, 1998). Excessive production of reactive oxygen species (ROS) has been implicated in MTX hepatotoxicity, nephrotoxicity, intestinal toxicity and cardiotoxicity (Abd El-Twab *et al.*, 2016; El-Sheikh *et al.*, 2016; Mahmoud *et al.*, 2017a,b). MTX negatively impact the mitochondrial machinery and therefore generates excess ROS (Kolli *et al.*, 2014). ROS can initiate peroxidation of the cell membranes and damage cellular macromolecules leading to cell death (Naik and Panda, 2007). In addition, inflammation has been reported to play a central role in MTX toxicity (Abd El-Twab *et al.*, 2016; Mahmoud *et al.*, 2017a,b). The nervous system has a high metabolic rate, high levels of polyunsaturated fatty acids and low levels of antioxidants; therefore, is vulnerable to damage by oxidative stress (Götz *et al.*,

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1994; Barnham *et al.*, 2004). Therefore, mitigating oxidative stress can prevent MTX toxicity to different body organs.

Berberine (BBR) is a natural isoquinoline alkaloid that can be isolated from *Coptis chinensis* (Teodoro *et al.*, 2013). Previous work from our lab showed the protective effect of BBR against oxidative stress in animal models of diabetes (Mahmoud *et al.*, 2017c) and hepatotoxicity (Germoush and Mahmoud, 2014; Mahmoud *et al.*, 2014; Mahmoud *et al.*, 2017b). Therefore, this study was designed to evaluate the protective effect of BBR against MTX-induced cerebral oxidative stress and inflammation in rats.

## MATERIALS AND METHODS

### Chemicals

MTX was supplied by Shanxi PUDE Pharmaceutical Company (Shanxi, China). BBR, reduced glutathione (GSH), 5,5'-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, pyrogallol, 2-(1-naphthylamino) ethylamine dihydrochloride, sulfanilamide, thiobarbituric acid and 1,1,3,3 tetramethoxypropane were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were supplied by standard suppliers.

### Experimental animals and treatments

Twenty-four male Wistar rats weighing between 130–150 g were used in the present investigation. The animals were obtained from the National Institute of Ophthalmology (Giza, Egypt) and were housed in well-aerated standard cages at normal atmospheric temperature ( $22 \pm 2^\circ\text{C}$ ) and normal 12 h light/dark cycle. They were given a free access to a standard pellet diet and water *ad libitum*. All animal procedures were approved by the Institutional Ethics Committee of Beni-Suef University (Egypt).

The animals were divided into 4 groups, each comprising 6 rats (N = 6) as following:

**Group I (Control):** rats received a single intraperitoneal (ip) injection of physiological saline and orally administered the vehicle 0.5% carboxymethyl cellulose (CMC) for 7 consecutive days.

**Group II (MTX):** rats received a single ip injection of MTX (20 mg/kg) dissolved in saline (Mahmoud *et al.*, 2017a) and orally administered 0.5% CMC for 7 consecutive days.

**Group III (MTX + 25 mg BBR):** rats received a single ip injection of MTX (20 mg/kg) and orally administered 25 mg/kg BBR (Mahmoud *et al.*, 2014) dissolved in 0.5% CMC for 7 consecutive days.

**Group IV (MTX + 50 mg BBR):** rats received a single ip injection of MTX (20 mg/kg) and orally administered 50 mg/kg BBR (Mahmoud *et al.*, 2014) dissolved in 0.5% CMC for 7 consecutive days.

### Samples collection and preparation

At the end of experiment, all rats were sacrificed under light ether anesthesia and the brain was quickly removed and rinsed with ice-cold saline. The cerebrum was dissected and kept

frozen in liquid nitrogen. Frozen cerebrum samples (10%w/v) were homogenized in cold phosphate-buffered saline, centrifuged and the clear supernatant was collected and stored at  $-80^\circ\text{C}$  to assay malondialdehyde (MDA), nitric oxide (NO), GSH, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST). Other samples from the cerebrum were collected on RNA later and kept at  $-80^\circ\text{C}$  for RNA isolation.

### Determination of lipid peroxidation, NO and antioxidant defenses

Lipid peroxidation was determined in the cerebrum homogenate by assaying the level of MDA according to the method of Preuss *et al.* (1998). NO level was assayed following the method of Grisham *et al.* (1996), and GSH content was determined according to the method of Beutler *et al.* (1963). SOD, GPx and GST were measured according to the methods of Marklund and Marklund (1974), Matkovic *et al.* (1998) and Mannervik and Gutenber (1981) respectively.

### Quantitative reverse transcriptase real time polymerase chain reaction (qRT-PCR)

To determine the effect of BBR on MTX-induced inflammation, the gene expression levels of nuclear factor-kappaB (NF- $\kappa$ B), inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF- $\alpha$ ) were assayed using PCR as we previously reported (Mahmoud, 2014). Total RNA was isolated from the frozen cerebrum samples using Trizol reagent (Invitrogen, USA). Isolated RNA was treated with DNase I (Thermo Scientific, USA) and quantified at 260 nm. RNA samples with A260/280 nm ratio of 1.8 or more were selected and the integrity was further confirmed using formaldehyde-agarose gel electrophoresis. Two  $\mu\text{g}$  RNA was reverse transcribed into first strand cDNA using RT kit (Thermo Scientific, USA). cDNA was amplified using SYBR green master mix (Thermo Scientific, USA) and the primer pairs listed in Table 1.

**Table 1:** Primers used in qRT-PCR.

Gene	GenBank Accession Number	Sequence (5'-3')	Amplicon Size (bp)
<i>NF-<math>\kappa</math>B</i>	NM_199267.2	F: TCTCAGCTGCGACCCCG R: TGGGCTGCTCAATGATCTCC	122
<i>iNOS</i>	NM_012611.3	F: ATTCCCAGCCCAACAACA R: GCAGCTTGTCAGGGATTCT	112
<i>TNF-<math>\alpha</math></i>	XM_008772775.2	F: AAATGGGCTCCTCTCATAGTTC R: TCTGCTTGGTGGTTTGTACGAC	111
<i>IL-1<math>\beta</math></i>	NM_031512.2	F: GACTTCACCATGGAACCCGT R: CAGGGAGGGAACACACGTT	200
<i>B-Actin</i>	NM_031144.3	F: AGGAGTACGATGAGTCCGGC R: CGCAGCTCAGTAACAGTCCG	71

The qPCR reactions included 10 min initial denaturation at  $95^\circ\text{C}$  and 40 cycles each consists of 30 sec denaturation at  $95^\circ\text{C}$ , annealing for 60 sec and extension at  $72^\circ\text{C}$  for 30 sec. The  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen, 2011) was used to analyze the amplification data and results were normalized to  $\beta$ -actin.

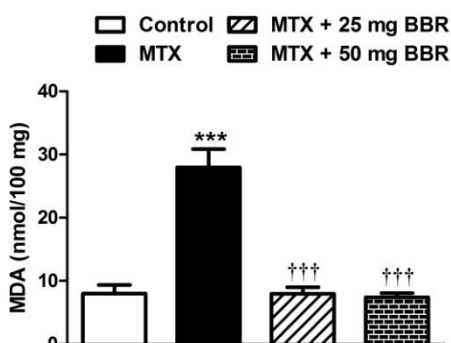
### Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Results were expressed as mean  $\pm$  standard error of the mean (SEM) and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test post hoc analysis. A *P* value  $<0.05$  was considered significant.

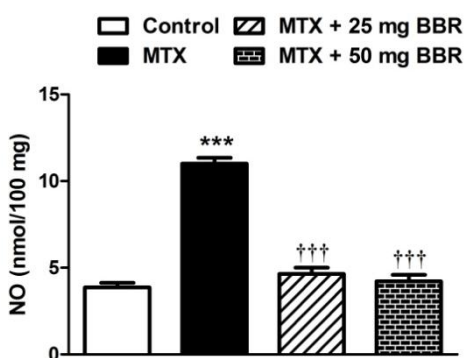
## RESULTS

### Berberine attenuates lipid peroxidation and NO generation in the cerebrum of MTX-induced rats

MTX administration induced a significant ( $P<0.001$ ) increase in the lipid peroxidation marker MDA in the cerebrum when compared with the control group (Fig. 1). Rats received 25 mg/kg body weight berberine showed a significant ( $P<0.001$ ) decrease in lipid peroxidation levels in the cerebrum. Similarly, MTX-induced rats treated with 50 mg/kg berberine exhibited significantly ( $P<0.001$ ) declined cerebral lipid peroxidation levels as depicted in Figure 1.



**Fig. 1:** Berberine attenuates lipid peroxidation in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P<0.001$  versus Control and ††† $P<0.001$  versus MTX. MDA, malondialdehyde; BBR, berberine.



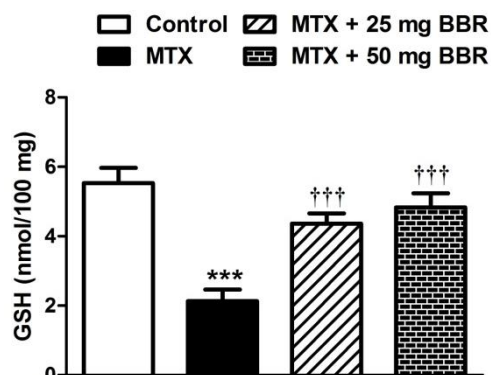
**Fig. 2:** Berberine reduces nitric oxide levels in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P<0.001$  versus Control and ††† $P<0.001$  versus MTX. NO, nitric oxide; BBR, berberine.

MTX-induced rats showed a significant ( $P<0.001$ ) increase in NO levels when compared with the control rats

(Fig. 2). Treatment with berberine at doses of 25 and 50 mg/kg body weight produced a significant ( $P<0.001$ ) decrease in cerebral levels of NO in MTX-administered rats when compared with the untreated MTX-induced rats (Fig. 2).

### Berberine increases GSH content in the cerebrum of MTX-induced rats

Analysis of the cerebral GSH content showed a significant ( $P<0.001$ ) decrease in MTX-induced rats when compared with the control group (Fig. 3). MTX-induced rats received 25 mg/kg body weight berberine showed a significant ( $P<0.001$ ) increase in cerebral GSH content. Similarly, the higher berberine dose significantly ( $P<0.001$ ) prevented MTX-induced GSH decline in the cerebrum of rats (Fig. 3).



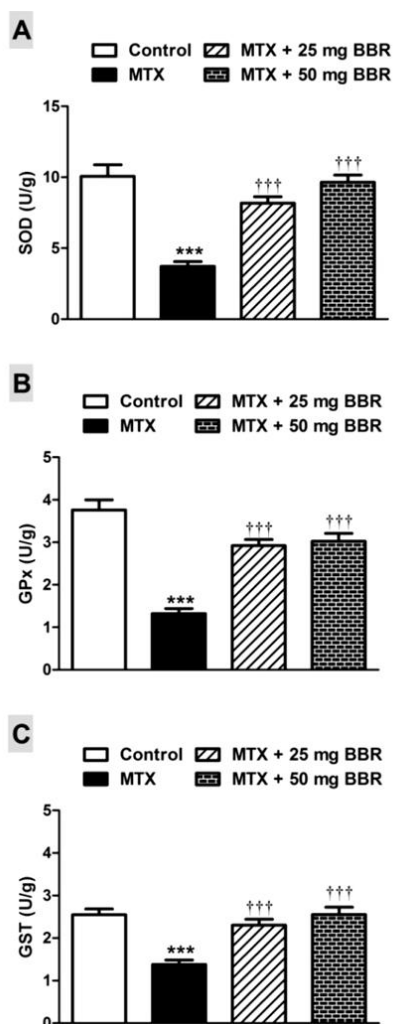
**Fig. 3:** Berberine increases reduced glutathione in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P<0.001$  versus Control and ††† $P<0.001$  versus MTX. GSH, reduced glutathione; BBR, berberine.

### Berberine enhances the activity of antioxidant enzymes in the cerebrum of MTX-induced rats

We determined the effect of berberine on the antioxidant defenses SOD, GPx and GST in the cerebrum of MTX-induced rats as represented in Figure 4.

MTX administration produced a significant ( $P<0.001$ ) decline in the activity of cerebral SOD when compared with the control group of rats (Fig. 4A). MTX-induced rats received either 25 or 50 mg/kg body weight berberine showed a significant ( $P<0.001$ ) increase in SOD activity.

GPx and GST showed a similar pattern where MTX-induced rats exhibited a significant ( $P<0.001$ ) decrease in their activity in the cerebrum. On the other hand, MTX-induced rats received berberine at doses of 25 and 50 mg/kg body weight showed significantly ( $P<0.001$ ) increased activity of cerebral GPx (Fig. 4B) and GST (Fig. 4C).

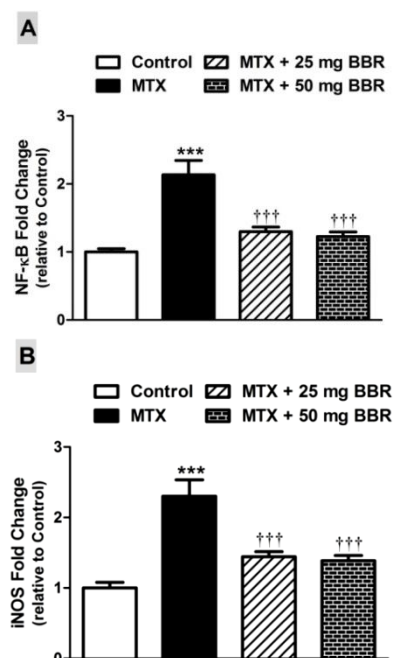


**Fig. 4:** Berberine enhances the activity of antioxidant enzymes in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P < 0.001$  versus Control and ††† $P < 0.001$  versus MTX. SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; BBR, berberine.

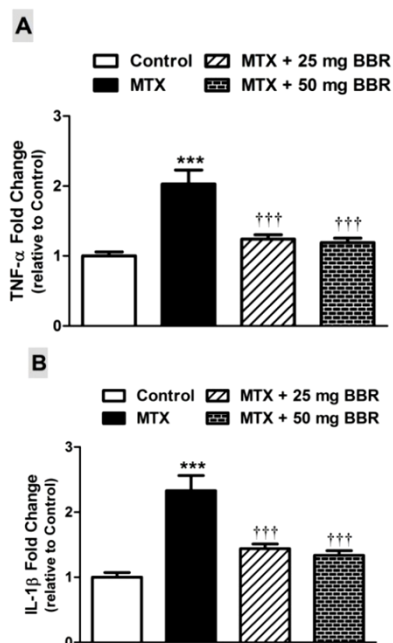
#### Berberine down-regulates NF- $\kappa$ B, iNOS and pro-inflammatory cytokines in the cerebrum of MTX-induced rats

To evaluate the protective effect of berberine against MTX-induced inflammation in the cerebrum of rats, we determined the gene expression levels of NF- $\kappa$ B, iNOS, TNF- $\alpha$  and IL-1 $\beta$ . Rats received MTX showed a significant ( $P < 0.001$ ) up-regulation in NF- $\kappa$ B gene expression when compared with the control rats as depicted in Figure 5A. Similarly, iNOS gene expression was significantly ( $P < 0.001$ ) up-regulated in the cerebrum of MTX-induced rats when compared with the control group (Fig. 5B). MTX-induced rats treated with berberine at doses of 25 and 50 mg/kg body weight showed a significant ( $P < 0.001$ ) down-regulation of NF- $\kappa$ B (Fig. 5A) and iNOS (Fig. 5B) mRNA expression in the cerebrum. Gene expression of the pro-inflammatory cytokines TNF- $\alpha$  (Fig. 6A) and IL-1 $\beta$  (Fig. 6B) showed a significant ( $P < 0.001$ ) increase in their mRNA levels in the cerebrum of MTX-induced rats when compared with the

control group. On the other hand, MTX-induced rats received 25 and 50 mg/kg body weight berberine exhibited a significant ( $P < 0.001$ ) down-regulation in mRNA expression levels of cerebral TNF- $\alpha$  and IL-1 $\beta$ .



**Fig. 5:** Berberine down-regulates (A) nuclear factor-kappaB (NF- $\kappa$ B) and (B) inducible nitric oxide synthase (iNOS) expression in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P < 0.001$  versus Control and ††† $P < 0.001$  versus MTX.BBR, berberine.



**Fig. 6:** Berberine down-regulates (A) tumor necrosis factor alpha (TNF- $\alpha$ ) and (B) interleukin-1beta (IL-1 $\beta$ ) expression in the cerebrum of MTX-induced rats. Data are Mean  $\pm$  SEM. Number of animals in each group is six. \*\*\* $P < 0.001$  versus Control and ††† $P < 0.001$  versus MTX.BBR, berberine.

## DISCUSSION

The present study demonstrated the protective effect of the natural isoquinoline alkaloid berberine on MTX-induced neurotoxicity in rats. Berberine prevented oxidative/nitrosative stress, enhanced the antioxidant defenses and mitigated inflammation in the cerebrum of MTX-induced rats.

Oxidative stress and surplus production of ROS have been reported to play a central role in the toxicity of MTX (Abd El-Twab *et al.*, 2016; El-Sheikh *et al.*, 2016; Mahmoud *et al.*, 2017a,b). The mechanism of excessive ROS generation by MTX involves its negative impact on the mitochondrial machinery (Kolli *et al.*, 2014) and declined antioxidant defenses (Abd El-Twab *et al.*, 2016; Mahmoud *et al.*, 2017a,b). Possible mechanisms of excessive ROS generation involve the increased activity of purine-catabolizing enzymes adenosine deaminase (ADA) and xanthine oxidase (XO). Impaired function of the mitochondrial membrane leads to impaired ATP metabolism with increased production of purine degradation products such as adenosine, inosine hypoxanthine and xanthine which are substrates for ADA and XO (Fadillioglu *et al.*, 2003). ROS are well-known to elicit peroxidation of the membranous lipids, damage DNA and proteins, and subsequently leading to cell death (Naik and Panda, 2007). The nervous system is particularly vulnerable to oxidative damage because of its high metabolic rate, high levels of polyunsaturated fatty acids and low levels of antioxidants (Götz *et al.*, 1994; Barnham *et al.*, 2004).

Here, MTX-induced rats showed a significant increase in cerebral lipid peroxidation and NO levels. NO can react with superoxide radical to produce the versatile and potent oxidizing agent peroxynitrite which induces further cell damage (McKim *et al.*, 2003). Interestingly, treatment with berberine markedly prevented MTX-induced lipid peroxidation and increased production of NO. In this context, berberine has been shown to protect against oxidative stress and memory impairment in a rat model of streptozotocin-induced diabetes (Bhutada *et al.*, 2011). In rats with nonalcoholic steatohepatitis, Ghareeb *et al.* (2015) reported the ability of berberine to reduce oxidative stress and neurotoxicity. In this study, berberine decreased lipid peroxidation and increased the antioxidant defenses in the brain tissue of rats. In addition, the study of Siow *et al.* (2011) demonstrated that berberine quenches superoxide anions and NO, and exerts radical scavenging activity against the high reactive peroxynitrite and hydroxyl radicals. In cell based systems, berberine showed a potent inhibitory effect on ROS production (Hur *et al.*, 2009) and prevented Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mediated superoxide production in lipopolysaccharide (LPS)-stimulated human monocyte-derived macrophages (Sarna *et al.*, 2010). Furthermore, Srikanth *et al.* (2011) reported that berberine inhibits NO production LPS-stimulated murine macrophages by inhibiting iNOS expression. Furthermore, previous work from our lab demonstrated that berberine suppressed lipid peroxidation and reduced NO levels in rat models of drug-induced liver injury (Germoush and Mahmoud, 2014;

Mahmoud *et al.*, 2014) and type 2 diabetes (Mahmoud *et al.*, 2017c). MTX administration significantly reduced the antioxidants GSH, SOD, GPx and GST in the cerebrum of rats. These findings support our recent studies where we reported declined non-enzymatic and enzymatic antioxidants in MTX-induced rats (Mahmoud *et al.*, 2017a,b). GSH is the most important cellular antioxidant for protecting against ROS and other oxidizing agents (Franco *et al.*, 2007). GSH is the substrate for GPx that works with SOD, GST and other enzymes to protect the cell against the deleterious effects of ROS (Wei *et al.*, 2011). The significant depletion of GSH induced by MTX administration might be the cause of reduced effectiveness of the antioxidant enzyme defense system, and therefore, sensitizing the cells to ROS (Babiak *et al.*, 1998). A recent study by Moore *et al.* (2016) demonstrated down-regulated SOD in different regions of the brain of methotrexate-treated rats. In addition, research from our laboratory showed a significant decline in GSH, SOD, GPx and GST in the kidney and liver of MTX-induced rats.

In the present study, MTX-induced rats received berberine exhibited significant amelioration in cerebral GSH, SOD, GPx and GST. Different studies have demonstrated the ameliorative effect of berberine on antioxidants in different tissue and organs in rats. We have previously reported that berberine enhances antioxidant defenses in liver of diabetic (Mahmoud *et al.*, 2017c) and cyclophosphamide- (Germoush and Mahmoud, 2014), isoniazid/rifampicin- (Mahmoud *et al.*, 2014) and MTX-induced rats (Mahmoud *et al.*, 2017b). Therefore, we assume that the antioxidant efficacy of berberine plays a central role in its neuroprotective effect. In this context, we have recently shown the potential of antioxidants to protect against neurotoxicity in rats (Mahmoud and Abd El-Twab, 2017).

In addition to oxidative stress, inflammation is a central contributing factor in MTX-induced toxicity. In the present study, administration of MTX induced a significant up-regulation of NF- $\kappa$ B and iNOS. NF- $\kappa$ B is a redox-sensitive transcription factor that can be activated as a result of increased ROS production (Matata and Galiñanes, 2002). Activated NF- $\kappa$ B elicits the transcription of genes of many inflammatory mediators such as iNOS, TNF- $\alpha$  and IL-1 $\beta$ . Here, the gene expression levels of iNOS, TNF- $\alpha$  and IL-1 $\beta$  were significantly up-regulated in the cerebrum of MTX-induced rats, demonstrating an inflammatory insult. In support of our findings, we have recently reported that the administration of MTX provoked inflammation in rats as evidenced by increased circulating levels of the pro-inflammatory cytokines (Abd El-Twab *et al.*, 2016; Mahmoud *et al.*, 2017a,b).

Berberine supplemented MTX-induced rats showed declined expression of NF- $\kappa$ B, iNOS, TNF- $\alpha$  and IL-1 $\beta$  in the cerebrum, pointing to a potent anti-inflammatory efficacy of berberine. Previous work from our laboratory showed that berberine prevents inflammation in diabetic (Mahmoud *et al.*, 2017c) and cyclophosphamide- (Germoush and Mahmoud, 2014), isoniazid/rifampicin- (Mahmoud *et al.*, 2014) and MTX-induced rats (Mahmoud *et al.*, 2017b). The antioxidant and anti-inflammatory mechanism of berberine involves activation of the

nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) as we previously demonstrated (Mahmoud *et al.*, 2014, 2017b,c). Nrf2 induces the transcription of antioxidative genes, (Jaiswal, 2004), and has been implicated in the control of inflammation. Nrf2 activation is associated with down-regulation of NF- $\kappa$ B and iNOS (Mahmoud and Al Dera, 2015) and decreased production of pro-inflammatory cytokines (Mahmoud and Al Dera, 2015, Kamel *et al.*, 2016; Mahmoud *et al.*, 2017a,b,d,e). In addition, PPAR $\gamma$  activation modulates the expression of several antioxidant genes (Girnun *et al.*, 2002, Chung *et al.*, 2009) and induce anti-inflammatory responses (Yu *et al.*, 2002).

In conclusion, the isoquinoline alkaloid berberine berberine prevented MTX-induced oxidative stress and inflammation in the cerebrum of rats. Berberine enhanced the non-enzymatic and enzymatic antioxidants in the cerebrum of MTX-induced rats. However, further studies are required to determine the exact neuroprotective mechanism of berberine.

**Financial support and sponsorship:** Nil.

**Conflict of Interests:** There are no conflicts of interest.

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#### How to cite this article:

Hozayen WG, Ramadan SM, Fadel A, Mahmoud AM. Berberine mitigates methotrexate-induced oxidative stress and inflammation in the cerebrum of rats. *J App Pharm Sci*, 2017; 7 (11): 043-049.