Neurotoxicology 72 (2019) 74–84

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Protective role of metformin against methamphetamine induced anxiety, depression, cognition impairment and neurodegeneration in rat: The role of CREB/BDNF and Akt/GSK3 signaling pathways

Saghar Keshavarzi[a], Sareh Kermanshahi[b], Leila Karami[b], Majid Motaghinejad[a,*], Manijeh Motevalian[c], Samira Sadr[d]

[a] Research Center for Addiction and Risky Behaviors (ReCARB), Iran Psychiatric Center, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Chemistry, Pharmaceutical Sciences Branch, Islamic Azad University (IUAPS), Tehran, Iran
[c] Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[d] Research and Development Department, Parsian-Exir-Aria pharmaceutical Company, Tehran, Iran

ARTICLE INFO

Keywords:
Methamphetamine
Metformin
Anxiety
Depression
CREB/BDNF
Akt/GSK3
Cognition impairment

ABSTRACT

Background: Methamphetamine is a neuro-stimulant with neurodegenerative effects, and ambiguous mechanism of action. Metformin is an antidiabetic agent with neuroprotective properties but not fully understood mechanisms. The present study investigated the molecular basis of metformin neuroprotection against methamphetamine-induced neurodegeneration.

Brief method: Sixty adult male rats were randomly divided into six groups: group 1 (received normal saline), group 2 (received 10 mg/kg of methamphetamine) and groups 3, 4, 5 and 6 [received methamphetamine (10 mg/kg) plus metformin (50, 75, 100 and 150 mg/kg) respectively]. Elevated Plus Maze (EPM), Open Field Test (OFT), Forced Swim Test (FST), Tail Suspension Test (TST) and Morris Water Maze (MWM) were used to assess the level of anxiety, depression and cognition in experimental animals. Also animals' hippocampus were isolated and oxidative stress and inflammatory parameters and expression of total and phosphorylated forms of cAMP response element binding (CREB), brain-derived neurotrophic factor (BDNF), protein kinase B (Akt) and glycogen synthase kinase 3 (GSK3) proteins were evaluated by ELISA method.

Results: According to the data obtained, methamphetamine caused significant depression, anxiety, motor activity disturbances and cognition impairment in experimental animals. Metformin, in all used doses, decreased methamphetamine induced behavioral disturbances. Also chronic administration of methamphetamine could increase malondialdehyde (MDA), tumor necrosis factor-Alpha (TNF-α) and interleukine-1 beta (IL-1β) in rats, while caused reduction of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities. Metformin, especially in high doses, could prevent these malicious effects of methamphetamine. Also Metformin could activate CREB (both forms), BDNF and Akt (both forms) proteins' expression and inhibited GSK3 (both forms) protein expression in methamphetamine treated rats.

Significance: According to obtained data, metformin could protect the brain against methamphetamine-induced neurodegeneration probably by mediation of CREB/BDNF or Akt/GSK3 signaling pathways. These data suggested that CREB/BDNF or Akt/GSK3 signaling pathways may have a critical role in methamphetamine induced neurotoxicity and/or neuroprotective effects of metformin.

1. Introduction

Methamphetamine (METH) is a central nervous system stimulant with high potential for abuse, without any clinical indication. But its adverse neurochemical and behavioral effects still need clinical clarification (Chang et al., 2007; Winslow et al., 2007). This agent shares functional and pharmacological characteristics with cocaine (Rusyniak, 2013; Winslow et al., 2007), and therefore confers an extremely high potential for abuse and addiction due to these similarities. Many previous studies showed that chronic administration of METH and its abrupt cessation can cause some neurobehavioral disturbances called METH withdrawal symptoms (Rusyniak, 2013; Winslow et al., 2007).

* Corresponding author.
E-mail address: dr.motaghinejad6@gmail.com (M. Motaghinejad).

https://doi.org/10.1016/j.neuro.2019.02.004
Received 26 February 2018; Received in revised form 2 February 2019; Accepted 5 February 2019
Available online 08 February 2019
0161-813X/

Also other studies claimed that the depletion of dopamine and norepinephrine, during long term uses of METH, and down regulation of receptors could be responsible for these behaviors (McConnell et al., 2015; Melo et al., 2012; Miyatake et al., 2005). Also other studies demonstrated that METH abuse can cause mitochondrial dysfunction, oxidative stress and inflammation which results in neurodegeneration in some brain areas but exact molecular mechanisms and signaling pathways involved in these malicious effects remain unclear (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009).

Metformin is a biguanide antidiabetic agent, prescribed in type 2 diabetes (El-Mir et al., 2008; Jiang et al., 2014). Previous studies have demonstrated that metformin has both anxiolytic and antidepressant effects, especially in diabetic patients. It may be a good alternative in situations where anxiety and depression both exist, as in the case of amphetamine and other psychostimulants abuse (El-Mir et al., 2008; Jiang et al., 2014). A large body of literature has demonstrated that this agent can be effective in modulation of neurodegeneration due to its anti-apoptotic and anti-oxidative properties (El-Mir et al., 2008; Ullah et al., 2012). Metformin can potentially act against some neurotoxins and molecular malicious effects of some neurodegenerative agents (Bergheim et al., 2006; El-Mir et al., 2008; Porceddu et al., 2016). It also seems to be effective in treatment of alcohol and METH and other forms of drug abuse (Bergheim et al., 2006; Porceddu et al., 2016). Many studies demonstrated that some protein kinases, such as AMP kinase, are involved in neuroprotective effects of metformin but its putative mechanism of action remains unclear (Fryer et al., 2002). On the other hand, some molecular studies demonstrated that effects of this AMP kinases are modulated by cAMP response element binding protein (CREB)/ brain-derived neurotrophic factor (BDNF) and protein kinase B (Akt)/glycogen synthase kinase 3 (GSK3) signaling pathways (Fryer et al., 2002; Lu and Xu, 2006). Also previous studies have shown that CREB acts as a major transcription factor in brain development and neurogenesis (Gonzalez and Montminy, 1989; Miletic et al., 2002). CREB is activated in a phosphorylated form and multiple protein kinases phosphorylate this transcription factor and convert CREB to its active form (Aguiar et al., 2011; Carlezon et al., 2005; Kitagawa, 2007; Réus et al., 2011; Terranova et al., 2016). CREB act on DNA and prompts the production of BDNF proteins which are important in neurogenesis and the development of neurons. Many previous works indicated that phosphatidylinositol 3-kinase (PI3K) can activate Akt in brain cells and by activation of this protein, glycogen synthase kinase 3 (GSK3) which is involved in neurodegeneration, will be inhibited protecting cells from neurodegenerative effects of GSK3 (Chen et al., 2004; Endo et al., 2006). Also previous works showed the role of Akt/GSK3 signaling pathway on cognitive activity (Chen et al., 2004). Because of importance of P-CREB/BDNF and Akt/GSK3 signaling pathways in modulation of neuroprotection, cognition and mood related behaviors, this study was designed to assess the role of these pathways in conferring neuroprotective effects of metformin against METH-induced oxidative stress, inflammation and alterations in neurobehavioral parameters. Therefore we examined the effects of metformin treatment on METH-induced deficits, depression and anxiety-like behaviors by OFT, FST, TST and cognition in the MWM tests, beside hippocampal P-CREB/BDNF and Akt/GSK3 protein levels, oxidative stress and inflammation biomarkers. The findings of present study make a better understanding of METH toxicity and the mechanisms involved.

2. Results

2.1. Results of anxiety and depressive behavior

2.1.1. Results of anxiety-like behavior in the Open Field Test (OFT) in control and treatment groups

As shown in Table 1, METH (10 mg/kg) treated animals had lower count of rearing number, lower rate of central square entries and also spent less time in the central square of the OFT in comparison to control group with F(5,42) = 4,970, F(5,42) = 7,461, F(5,42) = 4,779 respectively ($P < 0.001$ for central square entries and time spent in central square and $P = 0.0012$ for raring number). Our study indicated that metformin inhibited this effect of METH in a dose dependent manner and increased frequency of central square entries and also time spent in the central region of the OFT in METH treated groups. This difference was statistically significant in comparison to METH (10 mg/kg) only treated group with F (5,42) = 7,461 for central square entries and F (5,42) = 4,779 for time spent in the central region ($P < 0.001$) (Table 1). Also metformin just at highest dose (150 mg/kg) caused increase of rearing number in METH (10 mg/kg) treated animals. This increase was statistically significant in comparison to group treated by METH (10 mg/kg) only with F (5, 42) = 4,970 and $P = 0.0012$) (Table 1).

Table 1
The effects of various doses of metformin on open field anxiety like behavior in rats treated by 10 mg/kg of methamphetamine.

Group	Central square entries	Time spent in central square(sec)	Number of rearing
Control	25 ± 2	170 ± 9	14 ± 2
METH	12 ± 2.9[a]	119 ± 7[a]	4 ± 1[a]
METH + MF(50 mg/kg)	15 ± 1	141 ± 11[b]	7 ± 1
METH + MF(75 mg/kg)	16 ± 1.4[b]	145 ± 9[b]	7 ± 2
METH + MF(100 mg/kg)	24 ± 3[b]	153 ± 7 [b]	9 ± 1.5
METH + MF(150 mg/kg)	25 ± 1.7[b]	159 ± 9[b]	11 ± 1.6[b]

METH: Methamphetamine. MF: Metformin.
[a] Showed significant level with $P < 0.05$ vs. control group.
[b] Showed significant level with $P < 0.05$ vs methamphetamine only group.

2.1.2. Results of depressive-like behavior in Forced Swim Test (FST) in control and treatment groups

Animals in METH (10 mg/kg) treated group had less swimming time, with F (5, 40) = 17,69 and $P < 0.001$, and also longer immobility time, with F(5,42) = 122 and $P < 0.001$, compared to control group in FST (Table 2). Metformin, in all used doses, inhibited this effect of METH and increased swimming time, with F (5, 40) = 17.69 and $P < 0.001$, and decreased immobility time, with F (5, 42) = 122 and $P < 0.001$ in experimental animals; such changes were significant in comparison to the group received METH (10 mg/kg) only ($P < 0.001$) (Table 2). METH (10 mg/kg) significantly decreased the latency to immobility compared to control group with F (5, 43) = 6.494 and $P = 0.001$ (Table 2), While metformin, in all used doses, inhibited this effect of METH and increased the latency to immobility compared to METH (10 mg/kg) only treated group with F (5, 43) = 6.494 and $P = 0.001$ (Table 2).

2.1.3. Results of anxiety-like behavior in Elevated Plus Maze (EPM) in control and treatment groups

Control group spent more time in open arms, with F (5,

Table 2
The effects of various doses of metformin in Forced Swim Test (FST) depression-like behavior in rats treated by 10 mg/kg of methamphetamine.

Group	Time spent swimming (sec)	Time spent immobile (sec)	latency to immobility (sec)
Control	210 ± 7	34 ± 3	60 ± 6
METH	130 ± 8[a]	169 ± 4.3[a]	10 ± 7[a]
METH + MF(50 mg/kg)	154 ± 5[b]	128 ± 3[b]	30 ± 8[b]
METH + MF(75 mg/kg)	159 ± 5[b]	112 ± 3.9[b]	50 ± 7[b]
METH + MF(100 mg/kg)	178 ± 7[b]	98 ± 5[b]	40 ± 9[b]
METH + MF(150 mg/kg)	182 ± 7[b]	93 ± 4.5[b]	50 ± 5[b]

METH: Methamphetamine. MF: Metformin.
[a] Showed significant level with $P \leq 0.001$ vs. control group.
[b] Showed significant level with $P \leq 0.001$ vs methamphetamine only group.

Table 3
The effects of various doses of metformin anxiety-like behavior in Elevated Plus Maze (EPM) in rats treated by 10 mg/kg of methamphetamine.

Group	Time spent in open arm (sec)	Time spent in closed arm of (sec)	percent time in closed vs. open arms
Control	126 ± 5	105 ± 5	42% / 35%
METH	55 ± 6[a]	202 ± 9.4[a]	18% / 67%
METH + MF(50 mg/kg)	76 ± 4[b]	186 ± 4[b]	25% / 62%
METH + MF(75 mg/kg)	88 ± 5[b]	181 ± 4.9[b]	29% / 60%
METH + MF(100 mg/kg)	94 ± 6[b]	165 ± 7[b]	31% / 55%
METH + MF(150 mg/kg)	109 ± 5[b]	154 ± 6.5[b]	36.3% / 51%

METH: Methamphetamine. MF: Metformin.
[a] Showed significant level with $P < 0.001$ vs. control group.
[b] Showed significant level with $P < 0.001$ vs methamphetamine.

42) = 14.97, and less time in closed arms, with F (5, 42) = 28.38, in EPM in comparison to group under treatment by 10 mg/kg METH only ($P < 0.001$) (Table 3). Again treatment with metformin (50, 75, 100 and 150 mg/kg) significantly increased the presence of animals in open arms, with F (5, 42) = 14.97, and decreased the presence of animals in closed arms, with F (5, 42) = 28.38, in EPM as compared to the METH (10 mg/kg) only treated group ($P = 0.045$ for dose of 50 mg/kg of metformin and $P < 0.001$ for doses of 75, 100 and 150 mg/kg of metformin) (Table 3). Metformin in all mentioned doses could decrease the time spent in closed vs. open arms in METH treated rats (Table 3).

2.1.4. Results of anxiety-like behavior in Tail Suspension Test (TST) in control and treatment groups

Duration of immobility in METH (10 mg/kg) only treated group was significantly increased, with F (5, 42) = 28.38, compared to animals in control group in TST ($P \leq 0.016$). The immobility was abolished by metformin (75, 100 and 150 mg/kg) and this was statistically significant in comparison to animals received 10 mg/kg of methamphetamine only with F (5, 42) = 28.38 and $P \leq 0.021$ (Fig. 1).

2.2. Results of locomotor activity

2.2.1. Results of changes in ambulation distance in Open Field Test (OFT) in control and treatment groups

METH (10 mg/kg) treated animals have shown less ambulation distance in comparison to control group [F (5, 42) = 8.277, $P \leq 0.001$] (Fig. 2A). Metformin, with all used doses, increased the ambulation distance in METH treated rats and this increase was statistically significant in comparison to METH only treated group with F (5,

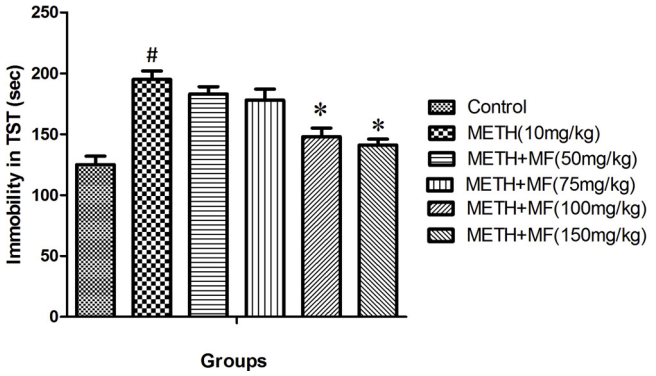

Fig. 1. Shows swimming time spent in immobility (seconds) in Tail Suspension Test (TST) in control group and groups treated with methamphetamine and 50, 75, 100 and 150 mg/kg of metformin in combination with methamphetamine. All data are expressed as Mean ± SEM (n = 8). # $P < 0.05$ vs. control group. * $P < 0.05$ vs 10 mg/kg of methamphetamine. METH: methamphetamine. MF: Metformin.

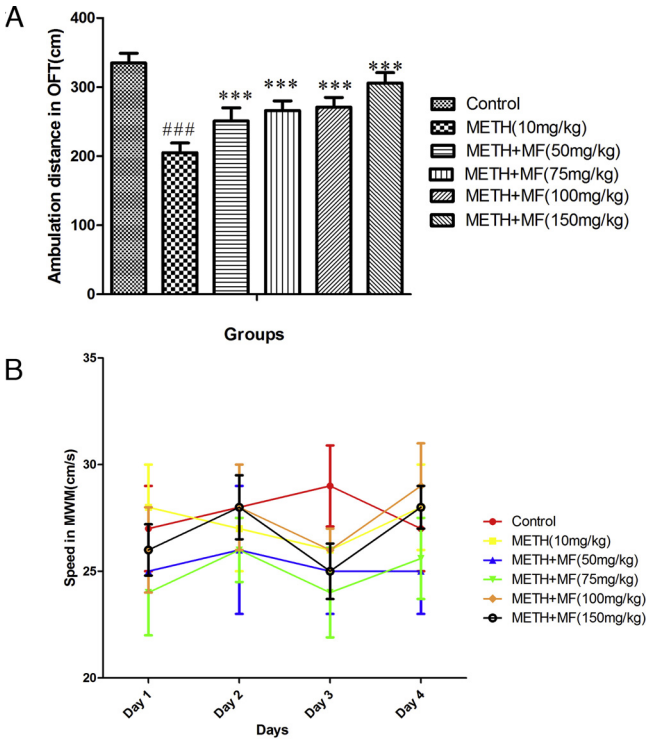

Fig. 2. Shows results of exploratory behavior and motor activity changes in Open Filed Test (OFT), Ambulation distance (2 A) and average of swimming speed in Morris Water Maze (MWM) (2B) in control group and groups treated by methamphetamine and 50, 75, 100 and 150 mg/kg of metformin in combination with methamphetamine. All data are expressed as Mean ± SEM (n = 8).
$P \leq 0.001$ vs. control group.
*** $P \leq 0.001$ vs 10 mg/kg of methamphetamine. METH: methamphetamine. MF: Metformin.

42) = 8.277 and $P < 0.001$ (Fig. 2A).

2.2.2. Results of changes in swimming speed during training days in Morris Water Maze (MWM)

The swimming speed was not altered during training trials in any of the animal groups, suggesting that exposure to METH (10 mg/kg) alone or in combination with metformin (50, 75, 100 and 150 mg/kg)did not cause any motor disturbance in experimental animals [(F (5, 42) = 0.3183, P = 0.9603)] (Fig. 2B).

2.3. Results of cognitive function

2.3.1. Evaluation of escape latency and traveled distance during training days in Morris Water Maze (MWM)

The change in escaped latency and traveled distance during four days training in the MWM for group under treatment by METH (10 mg/kg) was statistically significant compared to control group with F (5, 42) = 68.00 for escaped latency and F (5, 18) = 195.6 for traveled distance (Fig. 3A and B) ($P \leq 0.001$). Metformin (100 and 150 mg/kg) inhibited METH-induced increase in escape latency and traveled distance and this was significantly different compared to METH (10 mg/kg) only treated group with F (5, 42) = 68.00 for escaped latency and F (5, 18) = 195.6 for traveled distance (Fig. 3 A and B) ($P \leq 0.001$)

2.3.2. Evaluation of percentage in target quarter in probe trial in Morris Water Maze (MWM)

Results indicated that there was a significant decrease in percentage of the presence of animals in target quarter in METH (10 mg/kg) only treated group in comparison to control group with F (5, 42) = 5.801

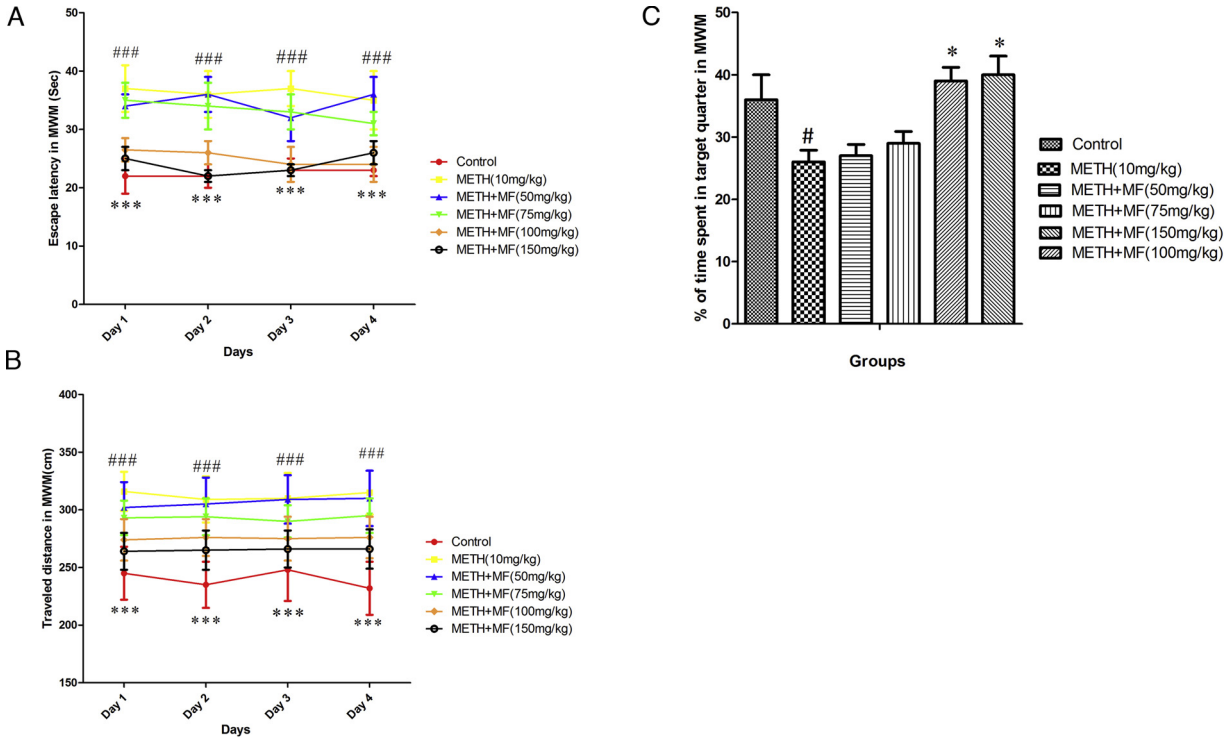

Fig. 3. Shows results of Morris Water Maze (MWM), average of escape latency (3 A), average of traveled distance (3B), percentage of time spent in target quarter in probe trial(3C) in control group and groups treated by methamphetamine and 50, 75, 100 and 150 mg/kg of metformin in combination with methamphetamine. All data are expressed as Mean ± SEM (n = 8).
P ≤ 0.001 vs. control group, # P < 0.05 vs. control group.
*** P ≤ 0.001 vs 10 mg/kg of methamphetamine. * P < 0.05 vs 10 mg/kg of methamphetamine
METH: methamphetamine. MF: Metformin.

and P = 0.004) (Fig. 3C). Also metformin with doses 100 and 150 mg/kg could diminish this effect of METH, but this effect was statistically significant in comparison to METH (10 mg/kg) only treated group with F (5, 42) = 5.801 and P = 0.004) (Fig. 3C).

2.4. Results of changes in biomarkers

2.4.1. Results of changes in oxidative stress biomarkers

METH administration significantly increased the lipid peroxidation as indicated by elevated mitochondrial malondialdehyde (MDA) level, with F (5, 42) = 18.33, and also decreased the superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities with F (5, 42) = 7.171, F (5, 42) = 9.467 and F (5, 42) = 7.008 respectively when compared to the control group (P ≤ 0.001) (Table 4). Conversely, various doses of metformin (50, 75, 100 and 150 mg/kg) reduced the METH-induced rise in MDA level and inhibited the METH induced decrease in SOD, GPx and GR activities when compared to the METH treated only rats with F (5, 42) = 18.33, F (5, 42) = 7.171, F (5, 42) = 9.467 and F (5, 42) = 7.008 respectively (P ≤ 0.001) (Table 4).

2.4.2. Results of changes in inflammatory biomarkers

The animals in METH only treated group demonstrated significant elevation in tumor necrosis factor-alpha (TNF-α), with F (5, 42) = 13.87, and interleukine-1beta (IL-1β), with F (5, 42) = 23.56 as compared to the control group (P ≤ 0.001) (Table 4). Conversely, high doses of metformin (100 and 150 mg/kg) prevented the METH-induced rise in pro-inflammatory biomarkers when compared to METH only treated animals with F (5, 42) = 13.87 for TNF-α and F (5, 42) = 23.56 for IL-1β (P ≤ 0.001) (Table 4).

Table 4
The effects of various doses of metformin on alterations of oxidative stress and inflammatory biomarkers in mitochondria of rats treated with methamphetamine (10 mg/kg/day).

Group	MDA nmol/mg of protein	SOD U/ml per mg of protein	GPx U/ml per mg of protein	GR U/ml per mg of protein	TNF-α ng/ml	IL-1β ng/ml
Control	8.4 ± 0.9	71.4 ± 6.1	78.2 ± 4.9	56.2 ± 5.3	49.4 ± 7.3	44.1 ± 3.1
METH(10 mg/kg)	29.8 ± 1.6[a]	34.2 ± 4.3[a]	35.2 ± 4.3[a]	19.1 ± 3.4	111.5 ± 5.9[a]	105.2 ± 4.1[a]
METH(10 mg/kg) +MF(50 mg/kg)	16 ± 1.2[b]	48.3 ± 5.2[b]	45.1 ± 7.3[b]	23.2 ± 5.2 [b]	89.5 ± 7.3 [a]	91.1 ± 5.1 [a]
METH(10 mg/kg) +MF(75 mg/kg)	12 ± 4 [b]	49.7 ± 5.2[b]	51.2 ± 7.1[b]	47.5 ± 7.3 [b]	83.2 ± 5.2	86.2 ± 4.1
METH(10 mg/kg) +MF(100 mg/kg)	10 ± 0.3 [b]	55.6 ± 4.6[b]	63.6 ± 5.2 [b]	47.4 ± 6.1 [b]	71.6 ± 4.6 [b]	80.1 ± 5.2 [b]
METH(10 mg/kg) +MF(150 mg/kg)	9.5 ± 0.6[b]	60.3 ± 6.2[b]	72.1 ± 2.9[b]	51.1 ± 7.1 [b]	69.2 ± 1.3 [b]	75.1 ± 2.1 [b]

METH: methamphetamine. MF: Metformin.
[a] Showed significant level with P ≤ 0.001 vs. control group.
[b] Showed significant level with P ≤ 0.001 vs methamphetamine only group.

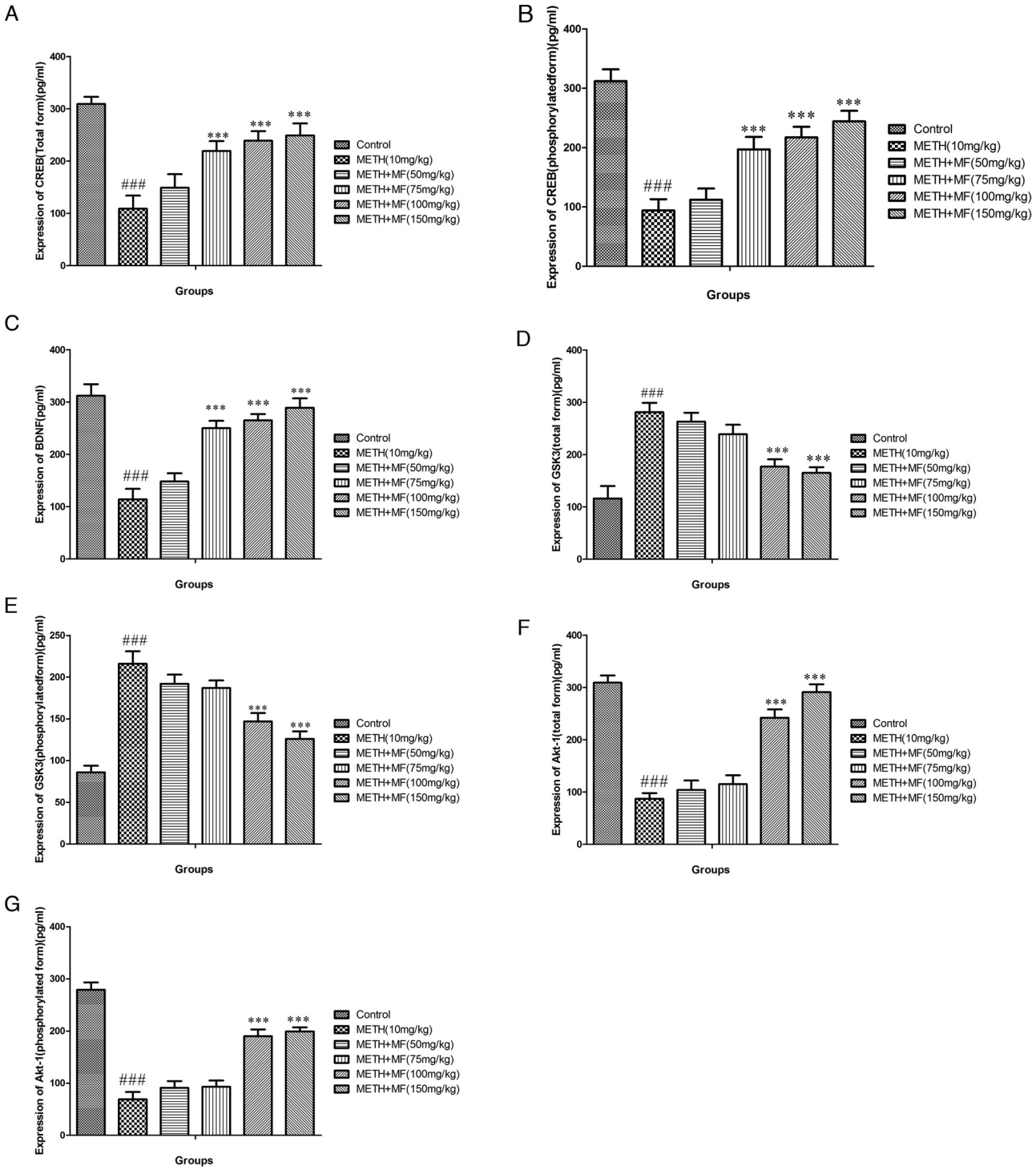

Fig. 4. Shows alterations of expression/level (ELISA) of CREB (total form)(4 A), CREB (phosphorylated form)(4B), BDNF (4C), GSK3 (total form) (4D), GSK3 (phosphorylated form) (4E), Akt (total form) (4 F), Akt (phosphorylated form)(4 G), in control group and group under treatment with methamphetamine and groups under treatment by methamphetamine in combination with metformin (50, 75, 100 and 150 mg/kg). All data are expressed as Mean ± SEM (n = 8).
P ≤ 0.001 vs. control group.
*** P ≤ 0.001 vs 10 mg/kg of methamphetamine. METH: methamphetamine. MF: Metformin.

2.5. The effects of metformin on METH-induced alterations in expression of CREB, P-CREB and BDNF proteins

METH (10 mg/kg) treatment markedly reduced the relative protein expression/level of P-CREB (total and phosphorylated) and BDNF in the rats' hippocampus in comparison to the control group with F (5, 42) = 11.57, F (5, 42) = 18.27 and F (5, 42) = 21.29 respectively (P ≤ 0.001) (Fig. 4A, B and C). Conversely, used doses of metformin (75, 100 and 150 mg/kg) significantly improved the protein expression/level of CREB (total and phosphorylated) and BDNF in METH-treated animals, with F (5, 42) = 11.57, F (5, 42) = 18.27 and F (5, 42) = 21.29 respectively, when compared to the METH only treated group (P ≤ 0.001) (Fig. 4A, B and C).

2.6. The effects of metformin on METH-induced alterations in expressions of both forms of Akt and GSK3 proteins

METH (10 mg/kg) treatment markedly reduced the relative protein expression/level of Akt (total and phosphorylated), with F (5, 42) = 43.11 and F (5, 42) = 43.32, and GSK3 (total and phosphorylated), with F (5, 42) = 13.5 and F (5, 42) = 20.86, in rats' hippocampus in comparison to the control group ($P \leq 0.001$) (Fig. 4D, E, F,G). Conversely, treatment with high doses of metformin (100 and 150 mg/kg) significantly improved the protein expression/level of Akt (total and phosphorylated), with F (5, 42) = 43.11 and F (5, 42) = 43.32, and GSK3 (total and phosphorylated), with F (5, 42) = 13.5 and F (5, 42) = 20.86, in METH-treated animals when compared to the METH only treated group ($P \leq 0.001$) (Fig. 4D, E, F and G).

3. Discussion

Current study showed that various doses of metformin can alter METH induced oxidative stress, inflammation and neurobehavioral parameters. According to our findings, metformin by modulation of CREB/BDNF and Akt/GSK3 signaling pathways could inhibit METH induced oxidative stress and inflammation and also behavioral changes such as anxiety, depression, and cognition impairment and motor activity disturbances. Previous studies demonstrated that abuse of METH can cause behavioral disorders and cognition impairment (Glasner-Edwards et al., 2010; McKetin et al., 2011). Also molecular studies showed that METH administration caused oxidative stress, neuro-inflammation and apoptosis, asserting that molecular malicious effects might be responsible for METH-induced neurodegeneration, but its exact mechanism and involved signaling pathways remain unclear (Cadet and Krasnova, 2009; Thrash et al., 2009). Metformin is a biguanide used in type 2 diabetes (El-Mir et al., 2008). According to previous studies and some powerful evidences, this agent has anxiolytic, antidepressant and cognition enhancer effects, especially in diabetic patients (Guo et al., 2014; Pintana et al., 2012; Zhao et al., 2014). Metformin also shows neuroprotective effect mediated by modulation of its anti-oxidant and anti-inflammatory activities, although the clear mechanism of action and involved signaling pathways have still to be defined (Wang et al., 2016; Zhao et al., 2014).

The result of current study showed that METH with dose of 10 mg/kg caused anxiety like behavior in OFT. Also data showed that metformin in all used doses decreased this type of anxiety-like behavior in OFT and inhibited the effects of METH on behavioral parameters (rearing number, central square entries and less time spent in the central square) in OFT. This part of data is consistent with pervious results which showed that METH abuse causes anxiety like behavior (Glasner-Edwards et al., 2010; Jablonski et al., 2015; McKetin et al., 2011). Previous study demonstrated that metformin can alter anxiety like behavior in OFT in rats and also probably can modulate anxiety like behavior which induced by drug abuse (Jablonski et al., 2015; Martin-Montalvo et al., 2013). According to present results, METH treatment induces a depressive like behavior and causes increase in immobility time, while decreases swimming time and latency to immobility in FST. These data are consistent with previous studies which showed that METH administration can cause depression in treated subjects (Glasner-Edwards et al., 2010, Li et al., 2013). Our results showed the modulation of METH induced changes in behavior by metformin in FST. Many previous clinical trials and experimental studies demonstrated that metformin, even with sub-therapeutic doses, can act as antidepressant and modulate depressive and anxiety like behaviors in diabetic depressed patients (Ge et al., 2017; Guo et al., 2014; Sarkaki et al., 2015). The present study indicates that administration of METH can induce occurrence of anxiety like behavior in EPM (causes decrease in time spent in open arms while increased time spent in closed arms) and in TST (caused increase immobility), while metformin, in all used doses, can decrease the anxiety like behavior in EPM and TST. These data are consistent with previous experimental and clinical studies which showed that METH and other similar compounds cause anxiety and stress related behaviors (Akindipe et al., 2014; Glasner-Edwards et al., 2010; McKetin et al., 2011). Also we can discuss these results with reference to the fundamental notion that metformin, and other similar neuroprotective agents are capable of alleviating anxiety- and stress-like behaviors in diabetic animals and during drug withdrawal period (Guo et al., 2014; Porceddu et al., 2016; Sarkaki et al., 2015). In consistency with our results, several previous studies have suggested that metformin can modify anxiety and depression in experimental tests and clinical trials especially in patient with metabolic syndrome (Guo et al., 2014; Martin-Montalvo et al., 2013; Sarkaki et al., 2015), but for the first time, we have found that metformin can alleviate anxiety and depression induced by METH treatment. It can be suggested that metformin, probably through its antidepressant and anxiolytic effects, can alter these behavioral disturbances in METH cessation.

Based on our study, chronic administration of METH at a dose of 10 mg/kg could decrease escaped latency and traveled distance during four days training and also caused decreased percentage of probe days presence in target quarter in MWM; this data showed that chronic administration of METH in mentioned doses can cause learning and memory impairment. Our data confirms the results of previous studies which showed that long-term administration of METH can modulate learning and spatial memory in immature rats (Akindipe et al., 2014; Hart et al., 2012; Jablonski et al., 2015). However, present data showed that metformin in used doses could affect METH-induced changes in cognition impairment. Many previous studies indicated that metformin have significant effect on learning and memory, and some others have demonstrated that this agent can enhance cognition (Guo et al., 2014; Martin-Montalvo et al., 2013; Pintana et al., 2012). According to current study METH administration can cause disturbance in motor activity (by decreases of ambulation distance) in OFT while it couldn't changes speed of animal in MWM. This data is inconsistent with previous studies which showed that METH-type stimulants can cause motor activity disturbance in OFT and decrease total ambulation distances in comparison to control subjects (Ares-Santos et al., 2014; Joca et al., 2014), while it doesn't affect total animal speed in MWM which there is still controversy about this issue and its effect on speed (Rau et al., 2016). Based on our data, our result showed that metformin in mentioned doses could decrease METH-induced motor activity disturbances in OFT while did not affect animal speed in METH treated animals in MWM. The role of metformin on motor activity disturbances did not show a direct correlation and related literature in this manner is poor but current data about protective role of metformin was discussable with potential role of metformin in management of anxiety and depression with indirect effect on motor activity (Guo et al., 2014; Martin-Montalvo et al., 2013; Pintana et al., 2012; Sarkaki et al., 2015). Overall, according to these behavioral results of our study, it can be suggested that metformin can act as an effective antidepressant, anxiolytic, cognitive enhancer and motor activity modulator in METH treated animals and can inhibit METH malicious effects on behaviour.

Consistent with our behavioral results, the molecular data demonstrated that METH by used dose can alter oxidative stress situation and neuro-inflammation. The present study indicated that METH can decrease SOD, GPx and GR activities while increasing MDA level as a marker of lipid peroxidation in rat hippocampus. Previous studies indicated that prolonged administration of METH caused mitochondrial dysfunction and alteration in respiratory chain anti-oxidative enzymes in brain cells of rodents; These studies suggested that METH can induce oxidative stress and lipid peroxidation in the brain of rats (Ares-Santos et al., 2014; Thrash et al., 2009). Nevertheless, the exact mechanism of action of METH and involved signaling pathways in this regard still remains unclear. According to previous studies, METH induced neurodegeneration was partly mediated by inactivation of antioxidant enzymes, activation of oxidative agents and lipid peroxidation (Thrash

et al., 2009; Walker et al., 2014). Result of current study demonstrated that treatment by metformin can be effective in reversing METH-induced increase in MDA level, as standard biomarker of lipid peroxidation, as well as reversing the reduction in SOD, GPx and GR activities in hippocampal tissues. The role of metformin in activation of antioxidant defense and increase the activity of antioxidant enzymes such as SOD, GPx and GR were approved by several studies (Ge et al., 2017; Hou et al., 2010; Porceddu et al., 2016; Sarkaki et al., 2015). These reports believed that metformin, as a highly potent antioxidant, can activate mentioned antioxidant enzymes and this type of activation could be involved in neuroprotection against some neurodegenerative situations especially in diabetic subjects (Ge et al., 2017; Hou et al., 2010; Porceddu et al., 2016; Sarkaki et al., 2015). SOD is an important enzyme that catalyzes the dismutation of the superoxide radicals into oxygen (O_2) or hydrogen peroxide (H_2O_2). GPx is another antioxidant enzyme that converts the malicious form of glutathione (GSSG) to its protective form (GSH), thus metformin can recover all mentioned antioxidant biochemical activity which disturbed by METH (Ge et al., 2017; Hou et al., 2010; Porceddu et al., 2016; Sarkaki et al., 2015). The inflammatory biomarkers such as TNF-α and IL-1β in the hippocampus were increased by METH administration. Previous similar studies also demonstrated that prolonged administration of METH increases pro-inflammatory markers, such as TNF-α and IL-1β, which is involved in METH induced neurodegeneration, but the signaling pathway and exact mechanism of action remains unclear (Loftis and Janowsky, 2014; Moratalla et al., 2014). On the other hand, we found that metformin can inhibit METH induced increase in TNF-α and IL-1β, as inflammatory biomarkers. Anti-inflammatory role of metformin and other similar agents have been already shown, and this property is the reason for its effectiveness in some neurodegenerative events especially in diabetic animals (Kim and Choi, 2012; Krasnova et al., 2016; Wang et al., 2016).

As we have noted, according to our study, metformin can inhibit METH induced neurobehavioral changes, oxidative stress and inflammation in hippocampal cells. This confirms previous results about neuroprotective effects of metformin (Kim and Choi, 2012; Wang et al., 2016). To define the molecular and signaling mechanisms of metformin neuroprotective action against METH induced behavioral and molecular malicious effects, we have tried to evaluate the molecular basis and possible signaling pathways involved in this regard. Thus we evaluated the CREB/BDNF and Akt/GSK3 signaling pathways. According to obtained data in current study, METH (10 mg/kg) can cause decrease in CREB and Akt protein level/expression in total and phosphorylated forms, also decreases BDNF. Also our data showed that METH can cause increase of GSK3 protein level/expression in total and phosphorylated forms. These data are in consistency with previous works which showed that METH type stimulants can inhibit phosphorylation of CREB and Akt in brain cells and by this inhibition of P-CREB, production of BDNF will be inhibited (Krasnova et al., 2013; Krasnova et al., 2016; Wu et al., 2015). Also by inhibition of phosphorylation of Akt, production and phosphorylation of GSK3 will be increased, which probably this GSK3 hyper-phosphorylation is responsible for METH induced neurodegeneration and neurobehavioral changes such as cognition impairment, anxiety and depression (Krasnova et al., 2013, 2016; Wu et al., 2015). Current study results showed that metformin especially in high doses can inhibit METH induced decreases of CREB protein level/expression in total and phosphorylated form, thus causes increases of BDNF production in METH treated rats. These effects of metformin were shown in doses 75, 100 and 150 mg/kg. Moreover according to our data, metformin especially in high doses (100 and 150 mg/kg), can inhibit METH induced increases in GSK3 and decreases in Akt protein level/expression. These data can further complete our knowledge about the metformin effects on signaling pathways and can confirm the role of metformin in activation of some protein kinases such as AMP kinase confirmed by previous studies (Hou et al., 2010; Huang et al., 2009; Jiang et al., 2014; Kim and Choi, 2012; Sarkaki et al., 2015). It has been shown by many previous studies that effects of some neuroprotective, cognitive enhancer and antidepressant agents against neurodegeneration were mediated by modulation of P-CREB/BDNF or P-Akt/P-GSK3 and other similar signaling pathways (Abreu-Villaça et al., 2018; Motaghinejad et al., 2017b; Seaborn et al., 2011; Srivastava et al., 2018), but the role of P-CREB/BDNF or P-Akt/P-GSK3 in metformin neurobehavioral and neurochemical protective effects have not been shown so far. According to the present findings, metformin and other similar agents might act through P-CREB/BDNF or P-Akt/P-GSK3 pathways in order to rescue cell survival and trigger neuroprotection. These novel findings give us a new insight in molecular aspects of metformin and METH effects in hippocampal cells.

4. Conclusion

From data obtained in present study, we can conclude that chronic administration of METH in adult rats causes behavioral and molecular changes which some parts of these neurodegenerative effects might be mediated by inhibition of production of P-CREB, BDNF and also inhibition of P-Akt, which will cause activation of GSK3. Also our data indicated that metformin can act against METH induced neurobehavioral changes and neurodegeneration, and for the first time, the current study showed that activation of P-CREB/BDNF and P-Akt and inhibition of P-GSK3 proteins signaling pathways might be involved in metformin protective effects against METH induced neurobehavioral and biochemical malicious effects. Although these findings give us a new insight about unknown mechanisms of metformin neuroprotection and METH neurodegenerative effects, but further evaluation of precise molecular and cellular aspects of metformin protective mechanisms against METH induced neurodegeneration and neurobehavioral changes seems necessary.

5. Materials & methods

5.1. Animals

Sixty adult male Wistar rats, weight 250–300 g, were obtained from animal house of Iran University of Medical Sciences. They were housed in a managed condition, room temperature (22 ± 0.5 °C) with 12-h light/dark cycles and had free access to food and water. The experimental protocol was approved by the Ethical Committee in Research Deputy of Iran University of Medical Sciences.

5.2. Experimental design

All animals were divided randomly into 6 groups (10 rats in each group).

- Group 1 (as control) was given normal saline (0.7 ml/rat, *ip*, once daily) for 21 days.
- Group 2 (as METH treated) received METH (10 mg/kg, *ip*, once daily) (Sigma-Aldrich, USA) for 21 days.
- Groups 3, 4, 5 and 6 concurrently were treated by METH (10 mg/kg, *ip*, once daily) and metformin (Sigma-Aldrich, USA) with doses of 50, 75, 100 and 150 mg/kg respectively for 21 days.

During days 22–28, some standard behavioral tests such as Elevated Plus Maze (EPM) on 22nd day, were done. Open Field Test (OFT), was done on day 24, Forced Swim Test (FST), was done on day 26 and Tail Suspension Test (TST) was done on day 28; Whole set of tests were used to investigate the level of anxiety and depression in experimental animals. In addition, a standard behavioral protocol by Morris Water Maze was applied to evaluate learning and spatial memory in treated animals between 17th and 21 st days. In day 29, all animals were anesthetized by 50 mg/kg thiopental and their brain tissues were removed and

hippocampus was isolated from each rat according to the guides present in previous studies (Motaghinejad et al., 2016). In order to investigate the behavioral changes in METH withdrawal period, the anxiety and depression were evaluated. For finding out the role of METH consumption on cognitive activity, concurrent evaluation of learning and spatial memory with METH and metformin treatment was implemented and its changes were compared with control group. It should be noted that hippocampus from right hemisphere was used for evaluation of oxidative stress and inflammation biomarkers but left hemisphere's hippocampus was used for evaluation of CREB, P-CREB, BDNF, Akt, P-Akt, GSK and P-GSK proteins expression.

5.3. Behavioral tests

5.3.1. Open field test (OFT)

This assay was used to evaluate anxiety and locomotor activity in experimental animals. The base of OFT apparatus was divided into 16 equally spaced squares bordered with opaque and 70 cm high walls. The whole apparatus was painted black except for the 6 mm broad white lines that divided the ground into 16 squares. This apparatus was illuminated using a 100 W bulb which focused into the field from a height of about 110 cm. Except for the open field; the entire room was kept in dark during the experiment. In order to observe subsequent behaviors for evaluating anxiety and locomotor activity, each animal was brought to the center of the setup for about 5 min;

1 Ambulation distance: Total distance of the grid lines crossed by each rat.
2 Center Square Entries: Number of times each rat enters the central red square lines with all four paws.
3 Center square duration: The time spent by each rat in the central square.
4 Rearing: Frequency with which each rat stands on their hind legs in the maze (Gould et al., 2009; Prut and Belzung, 2003; Sudakov et al., 2013).

5.3.2. Forced swim test (FST)

This is a behavioral test used for the evaluation of depression-like behaviors in the experimental model. The FST equipment consisted of a transparent plexiglas cylinder (20 cm diameter × 60 cm height) filled with water up to the height of 30 cm from base. A day before the test, all rats were gently placed in cylinder and made to swim for a habituation period of 15 min. However during experimentation, subjects were placed individually in filled glass cylinder for a period of 5 min and duration of swimming was recorded for each. The swimming activity was indicative of non-depressive behavior (Motaghinejad et al., 2016; Petit-Demouliere et al., 2005).

5.3.3. Elevated plus maze (EPM)

EPM is a widely used assay to assess anxiety-related behaviors in rodents. EPM setting consists of a plus sign apparatus; a pair of oppositely placed open arms (55 × 15 cm), a pair of oppositely placed closed arms (55 × 15 cm) and an open central squared area (10 × 10 cm). All arms were kept open from top and closed arms had 40 cm elevated side walls. The entire apparatus was held 50 cm above the ground by means of rods. One at a time, rats were placed in the center of the maze and the time spent by each rat in open arms and in closed arms was recorded. The subjects were freely allowed to explore the maze for 5 min. The preference for being in closed arms over the open ones was indicative of depressive behavior (López-Crespo et al., 2009; Sudakov et al., 2013).

5.3.4. Tail suspension test (TST)

In TST, a rat is suspended by its tail (50 cm above the ground) against a fixed metal rod with its body facing downwards. Normally, the rat tries to escape from this stressful state by trying to climb up the metal rod. However, depressed rats remain immobile. Therefore, we recorded duration of immobility in a 5-minute period which was indicative of depressive-like behavior (Ripoll et al., 2003).

5.3.5. Morris Water Maze task (MWM)

MWM apparatus includes a black colored circular tank, filled with water, 160 cm in diameter and 90 cm in height, being fixed in the center of the experimental lab. This equipment was divided into four quadrants (North, East, West, and South) and was filled with water to the height of 50 cm. The operator stays in the North-East part of the room. A disk on the platform with 15 cm diameter, in a hidden status, was located 1 cm beneath the surface of the water. In the first 4 days of the experiment, which is called training period, mentioned platform was randomly inserted persistently in one of the quarters. An automated infrared tracking system (CCTV B/W camera, SBC-300 (P), Samsung Electronics Co, Ltd, Korea) recorded the position of the animal in the tank. The camera was mounted 2.4 m above the surface of the water (D'Hooge and De Deyn, 2001; Motaghinejad et al., 2015d).

5.3.5.1. Handling. On the first day before the start of the experiment, all rats one by one were positioned on in the tank that was filled with 40 °C water, room temperature (25 ± 2 °C) and the experimenter guided the rat to swim and reach the quarter where platform was placed. In our experiment, the platform was situated on South-East quarter of a tank (D'Hooge and De Deyn, 2001; Motaghinejad et al., 2015d).

5.3.5.2. Training procedure. Some discriminate landmarks (such as a distinguished picture, window, door, etc.) were placed in the extra maze inside the room for spatial cues to be learnt about the platform's position for animals. As mentioned above, the position of platform was set up in the South-East quarter of the MWM tank with 25 cm distance from tank edge, and 1 cm beneath the surface of the water. For evaluation of learning procedure, each rat took part in 4 trials per day for 4 days. Each animal was randomly placed in four quarters (North, East, West, and South) respectively. During the learning procedure, if the rats found the platform within the 60 s, the trial would be automatically closed by a computer but if they could not reach and found the platform within 60 s, the trial would automatically be stopped by computer. In learning experiment, two parameters were evaluated:

1. The time period of escape latency characterized by time to find the hidden platform.
2. Traveled distance which was defined as the distance each animal spent to reach and find the hidden platform.

In spatial memory assessment procedure, on the fifth day (probe day), the platform was removed and animal was randomly terrified of the water from one of the above-mentioned directions (almost East). The percentage of animal presence in target quarter (South-East quarter) was recorded and calculated (Bromley-Brits et al., 2011; D'Hooge and De Deyn, 2001; Motaghinejad et al., 2015a,d; Vorhees and Williams, 2006).

5.4. Molecular studies

5.4.1. Mitochondrial preparations

Animals were anesthetized using sodium thiopental (50 mg/kg, i.p) and hippocampus was dissected from each rat. The isolated tissues were homogenized in cold homogenization buffer (25 mM 4-morpholine-propanesulfonic acid, 400 mM sucrose, 4 mM magnesium chloride ($MgCl_2$), 0.05 m methylene glycol tetra-acetic acid (EGTA), pH 7.3) and the homogenized tissues were centrifuged at 450 × *g* for 10 min. Obtained supernatants were re-centrifuged at 12,000 × *g* for 10 min. Finally, the sediments were re-suspended in homogenization buffer and stored at 0 °C. Total mitochondrial proteins in tissues were determined using a Dc protein assay kit (Bio-Rad), (California, USA). Briefly;

Bradford reagent (1 part Bradford: 4 parts dH_2O) was added to serial dilution series (0.1–1.0 mg/ml) of a known protein sample concentration; e.g., bovine serum albumin (BSA), dissolved in homogenization buffer. These serial dilution series were prepared and used for providing a standard curve. On the other hand 10, 15, 20, 25 and 30 µl of the protein extract (homogenized cell solutions) were added to multiple wells. Bradford reagent was also added to each well. The density of colors of all wells was read by the plate reader at 630 nm. Finally, by using the standard curve, protein quantity in the extracts was obtained. These homogenized cell solutions, containing mitochondria of hippocampal cells, were analyzed for the measurement of oxidative stress and inflammatory markers (Motaghinejad et al., 2015c, 2017c,d, 2016; Picard et al., 2011).

5.4.2. Measurement of oxidative stress parameters

5.4.2.1. Determination of lipid peroxidation. For assessment of lipid peroxidation, malondialdehyde (MDA) - a natural by-product was assessed. Briefly, 100 µL of SDS lysis solution was added to wells containing (100 µL) of sample solution or MDA standard. After shaking and incubation of these wells, 250 µL of thiobarbituric acid (TBA) reagent was added to each well and incubated at 95 °C for 45–60 min. Then tubes were centrifuged at 1000 × *g* for 15 min and 300 µl of n-Butanol was added to 300 µL of the supernatant. Next the tubes were centrifuged for 5 min at 10,000 × *g*. Finally, the absorbance was read at 532 nm and the obtained results were expressed as nmol/mg of protein (Motaghinejad et al., 2015c, 2017a,b,c,d, 2016; Papastergiadis et al., 2012; Picard et al., 2011).

5.4.2.2. Study of manganese superoxide dismutase (SOD) activity. In order to determine the SOD activity, 20 µL of unknown sample solution was added to each sample and 2nd blank wells, and 20 µL of ddH2O (double distilled water) was added to 1st and 3rd blank wells. Then, 200 µL of WST Working Solution (1 ml of water-soluble tetrazolium salt; WST dissolved in 19 ml of Buffer Solution) was added to each well and mixed. 20 µL of Dilution Buffer was added to 2nd and 3rd blank wells. Furthermore, 20 µL of enzyme working solution to was added to each sample and 1st blank well. After mixing thoroughly, the plates were incubated at 37 °C for 20 min and absorbance was read at 450 nm using a microplate reader. As recommended by manufacturer, SOD activity was calculated using the following equation: SOD activity = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} × 100. Data was reported as U/ml/mg protein (Weydert and Cullen, 2010; Zhang et al., 2009).

5.4.2.3. Determination of glutathione peroxidase (GPx) activity. For assessment of GPx activity, 20 µL of sample (which was diluted in advance by assay buffer) or assay buffer alone was added to sample and its corresponding well. Following this, 200 µL of reaction solution was added to each well. After setting up the microtiter plate reader at 340 nm over a time period of 8 min at 25 °C, 20 µL of peroxide substrate solution was added to each well and absorbance was measured. For data evaluation, delta OD between minutes 2–8 was used. As recommended by the manufacturer, change in absorbance [ΔA_{340}/ min] was calculated by following equation:

ΔA340/min = A340 nm (Start) – A340 nm (Stop)/ Reaction time (min), any change in absorbance is directly proportional to GPx activity (Weydert and Cullen, 2010; Zhang et al., 2009).

GPx activity: ΔA340/min × Reaction volume (ml) × Dilution factor of the original sample / Extinction coefficient for NADPH at 340 nm × Volume of the tested sample. Results were expressed as mU/mg protein (Weydert and Cullen, 2010; Zhang et al., 2009).

5.4.2.4. Determination of glutathione reductase (GR) activity. To assess GR activity, 25 µl of the sample (which was diluted beforehand with the assay buffer) or assay buffer alone was added to the sample and its corresponding well and then, 250 µl of the special reaction solution was added to each well according to manufacturer instructions. Then, micro plates were read at 340 nm. The OD of sample wells was inserted in standard curve which was drawn previously by manufacture kits. The activity of GR in unknown sample solution was measured by insertion of OD and calculation of enzyme activity. Results were expressed as mU/mg protein (Motaghinejad et al., 2015b,c; Shamoto-Nagai et al., 2007).

5.4.3. Measurement of protein expression

5.4.3.1. Determination of alterations in protein expression. Concentration (of expressed protein) for CREB (total and phosphorylated), BDNF, Akt1 (total and phosphorylated), GSK3 (total and phosphorylated), TNF-α and IL-1β in cell lysate of hippocampal tissue, were measured by using a commercially available ELISA kit (Genzyme Diagnostics, Cambridge, U.S.A). Briefly, wells containing sheep anti-rat BDNF,CREB (total and phosphorylated), IL-1β and TNF-α polyclonal antibodies (Sigma Chemical Co., Poole, and Dorset, UK) were washed three times with washing buffer (0.5 M of Sodium chloride (NaCl), 2.5 mM sodium dihydrogen phosphate (NaH_2PO_4), 7.5 mM Na_2HPO_4, 0.1% Tween 20, pH 7.2). Then, 100 ml of 1% (w/v) ovalbumin (Sigma Chemical Co., Poole, Dorset, UK) solution was added to each well and incubated at 37 °C for 1 h. Following three washes, 100 µl of samples and standards were added to each well and incubated at 48 °C for 20 h. After three washes, 100 µl of the biotinylated rabbit anti-rat CREB (total and phosphorylated), BDNF, Akt1 (total and phosphorylated), GSK3 (total and phosphorylated), TNF-α and IL-1β antibodies (1:1000 dilutions in washing buffer containing 1% sheep serum, Sigma Chemical Co., Poole, and Dorset, UK) was were added to each well. Next, after 1-hour incubation and three washes, 100 µl avidin-HRP (Dako Ltd, UK) (1:5000 dilution in wash buffer) was added to each well and the plate was incubated for 15 min. After washing three times, 100 µl of TMB substrate solution (Dako Ltd., UK) was added to each well and then incubated for 10 min at room temperature. Then, 100 µl of 1 M H_2SO_4 was added and absorbance was read at 450 nm. Results were expressed as ng for IL-1β/ml or TNF-α/ml of suspension of hippocampus tissues and about the for CREB (total and phosphorylated), BDNF,Akt1 (total and phosphorylated), GSK3 (total and phosphorylated) were reported as pg/ml of suspension of hippocampus tissues (Arican et al., 2005; Demircan et al., 2006; Lee et al., 2007; Shi et al., 2010).

5.5. Statistical analysis

The data were analyzed by Graph Pad PRISM v.6 Software and averaged in every experimental group and expressed as means ± standard error of the means (SEM). Then the differences between control and treatment groups were evaluated by ANOVA. Also in MWM test repeated ANOVA measures were used for comparison of behavior during four days training and their differences were evaluated in each day. To evaluate the severity of behaviors, the differences among averages in each group were compared using the Tukey's post-test at a significant level of $P < 0.05$ and exact P value was reported, but about the differences with $P < 0.001$ or less, the differences were reported as $P < 0.001$ as a replacement to exact P value.

Conflict of interest

There is no conflict of interest

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