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Research Paper

Neuroprotective Effects of Berberine Hydrochloride on Methamphetamine-induced Cognitive Dysfunction: Immunohistochemical and Behavioral Studies in Rats

Leila Rezaeian[1], Mehdi Khaksari[2], Raheleh Rafaiee[3], Hamid Kalalian Moghaddam[4*]

1. Department of Addiction Studies, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.
2. Addiction Research Center, Shahroud University of Medical Sciences, Shahroud, Iran.
3. Department of Neuroscience, School of Advanced Technologies in Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
4. Department of Physiology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.

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ABSTRACT

Introduction: Methamphetamine (MA) as an addictive psychostimulant drug affects the central nervous system. The current research aimed to evaluate the impact of berberine hydrochloride on improving cognitive function and neuroprotective effects in rats addicted to MA.

Methods: In this study, 27 male Wistar rats were randomly assigned to three groups, including control, MA addiction, and MA addiction with berberine hydrochloride (100 mg/kg/d) orally during the three weeks of withdrawal. Two groups received self-administered inhaled MA for two weeks (up to 10 mg/kg). Following the experimental procedures, a Morris water maze (MWM) and shuttle box were used to assess memory, and hippocampal sections from the animals were examined for caspase-3, Ki-67, and glial fibrillary acidic protein (GFAP) expression.

Results: The obtained results from the Morris water maze (MWM) showed that berberine hydrochloride decreases ($P<0.01$) the distance moved and the time spent to reach the hidden platform in the four-day learning trails phase and significant differences were observed in the distance moved, spent time, and frequency of motion in target quadrant on probe test day between groups. Berberine hydrochloride also reduced the latency of animals entering the dark chamber in the treated group compared to the control group ($P<0.05$). A significant decrease in activation of caspases-3, higher percentages of Ki-67 expression, and an increase in glial fibrillary acidic protein (GFAP) expression of cells was observed in the addicted group compared to the berberine-treated and control groups ($P<0.05$).

Conclusion: Administration of berberine hydrochloride for 3 weeks improves cognitive function in MA addiction and it has potential neuroprotective efficacy.

*** Corresponding Author:**
Hamid Kalalian Moghaddam, PhD.
Address: *Department of Physiology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.*
Tel: *+98 (912) 3731855*
E-mail: *h.kalalian@shmu.ac.ir*

Highlights

- Methamphetamine (MA) as an addictive psychostimulant drug affects the central nervous system.
- The berberine hydrochloride effects on improving cognitive function and neuroprotective.
- No approved pharmacotherapy, as well as confirmed medication, is available to treat MA abuse.

Plain Language Summary

Methamphetamine (MA) is known as a strong addictive stimulant with high addiction and no approved pharmacotherapy, as well as confirmed medication, is available to treat MA abuse. The study on the long-term effect of MA exposure on cognitive function during an object recognition memory test showed cognitive dysfunction after MA exposure. Berberine can reduce induced amnesia, which can be due to the increased peripheral and central cholinergic neuronal system functions, in addition, the most important mechanism in the protective effect of berberine against amnesia is the inhabitation of inflammation; however, the berberine impact on cells should be more investigated.

1. Introduction

Methamphetamine (MA) is known as a strong addictive stimulant with high addiction liability (Volkow & Nora 2014). The short-term health effects of MA are characterized by increased wakefulness and energy, irregular heartbeat, increased blood pressure, abnormally rapid breathing, high body temperature, and loss of appetite, and long-term misuse of MA have many negative consequences, including addiction, cardiovascular risks, and severe mental disorders. Psychotic symptoms of MA include anxiety, confusion, insomnia, paranoia and visual hallucinations, deterioration of attention, memory loss, and violence, reflecting significant changes in the brain (Winslow, Voorhees, & Pehl, 2007). MA as an addictive psychostimulant drug affects the central nervous system by altering the activity of the extracellular monoamine neurotransmitters (dopamine, serotonin, norepinephrine) by promoting their release from nerve terminals. MA increases extracellular dopamine levels through dopamine reverse transport by competing with dopamine uptake from vesicular stores and blocks its synaptic reuptake (King & Ellinwood, 1997). MA also impacts noradrenergic, serotonergic, and glutamatergic systems, N-methyl-D-aspartate receptors, and monoamine transporters. In many studies, acute and chronic MA users have shown severe functional and structural alterations in brain regions involved in deficits in memory and emotion. Those with MA abuse have significantly lower cognitive scores compared to the non-abusing controls (Scott et al., 2007).

The study on the long-term effect of MA exposure on cognitive function during an object recognition memory test showed cognitive dysfunction after MA exposure in rats. For example, when rats were exposed to high MA doses one week following MA administration, they showed cognitive impairments, such as object recognition memory (Belcher, O'Dell, & Marshall, 2005; Scott et al., 2007; Siegel, Craytor, & Raber, 2010), spatial, reversal and sequential learning (Acevedo, de Esch, & Raber, 2007; Chapman, Hanson, Kesner, & Keefe, 2001; Daberkow, Kesner, & Keefe, 2005; Kosheleff, Rodriguez, O'Dell, Marshall, & Izquierdo, 2012; Vorhees, Skelton, & Williams, 2007) and working memory (Dean, Groman, Morales, & London, 2013; Mizoguchi & Yamada, 2011).

No approved pharmacotherapy, as well as confirmed medication, is available to treat MA abuse (Karila et al., 2010). Berberine Hydrochloride is an isoquinoline plant alkaloid originally gained from rhizoma Coptidiscoptidis, Hydrastis Canadensis, and berberis aquifolium with antibiotic, anticancer, anxiolytic, antiamnesic, analgesic, anti-inflammatory, and antipsychotic and antidepressant activities (Kulkarni & Dhir, 2010; Meeran, Katiyar, & Katiyar, 2008; Moghaddam, Baluchnejadmojarad, Roghani, Goshadrou, & Ronaghi, 2013; Tillhon, Guamán Ortiz, Lombardi, & Scovassi, 2012; N. Wang et al., 2010; Wojtyczka et al., 2014; Zhang et al., 2008). Berberine can reduce induced amnesia, which can be due to the increased peripheral and central cholinergic neuronal system functions (Baradaran, Rabiei, & Rafieian, 2012). In addition, the most important mechanism in the protective effect of berberine against amnesia is the inhabitation of inflammation (Mohammadzadeh, Mehri, & Hosseinzadeh, 2017). Studies to date have shown that berberine significantly decreases pro-inflamma-

tory cytokine synthesis (Lee, Hyun, & Kim, 2010), and the glial fibrillary acidic protein (GFAP) expression, a marker for astrocyte activation (Kim et al., 2019) and decreases apoptosis through caspase-3 activation (C.-C. Lin, Kao, Chen, Ho, & Chung, 2006). However, the berberine impact on cells should be more investigated. The present research aimed to evaluate the impact of administration of Berberine Hydrochloride on MA-induced cognitive dysfunction and brain inflammation by cell proliferation, inflammation, and apoptosis agents in the hippocampus.

2. Materials and Methods

Animals

A total of 27 male Wistar rats (200-250 g) were provided from Pasteur Institute (Tehran, Iran) and were placed in a room (21°C±3°C) under 12h light and 12h darkness. Food and water were freely accessible. The experiments were performed based on the National Institutes of Health Guideline for the care and use of laboratory animals. Studies were performed from 8 AM to 12 AM. The samples were then randomly divided into three groups:

1. Control group: untreated intact animals (n=8)

2. Addicted group: the animals in this group (n=7) received 14 days of inhaled MA followed by 14 days of drug abstinence.

3. Berberine-treated group: the animals in this group (n=12) received 14 days of inhaled MA and daily oral gavage of berberine (100 mg/kg) during the three weeks of withdrawal. Berberine (Sigma-Aldrich; Merck Millipore, Germany) was prepared by dissolving in saline (Alavijeh, Vaezi, Khaksari, & Hojati, 2019).

Two groups received self-administration MA for two weeks with comprehensive modeling machine (made with Noavaran Sanaye Amouzeshi, Mashhad, Iran). Methamphetamine hydrochloride (Sigma-Aldrich; Merck Millipore, M8750, USA) was dissolved in distilled water in the first week of addiction a concentration of 1 mg/cc (5 mg/kg), and in the second week, at a concentration of 2 mg/cc (10 mg/kg) that we described in our previous paper (Rafaiee et al., 2019).

At the end of the experiment, memory was assessed using Morris water maze (MWM) and shuttle box and hippocampal sections of the animals were examined for caspase-3, Ki-67, and GFAP expression. Behavioral tests were performed in all animals of each group and four animals per group were used for immunofluorescent staining (Figure 1).

Morris Water Maze (MWM) Test

MWM assesses spatial memory (Asi et al., 2011). MWM included a dark circular pool filled with water (32 cm) at 22°C±1°C with 60 cm in high and 150 cm in diameter. The circular water tank was partitioned into four equal quadrants consisting of four starting positions of south, west, north, and east, and a clear platform of 10 cm in diameter was located in the center of the northern part, two centimeters below the surface of the water. The platform provided the only escape from the water. The position of the rats was monitored by a video camera attached to a computer mounted directly above the center of the MWM pool to record the distance moved (cm) to reach the hidden platform, the velocity (cm/s), and the time spent (s) in the target quadrant in samples. Animals were trained for 4 consecutive days at nearly the same time and received 4 tests per day their memory was tested on the 5th day, during which the platform was removed (probe test). In the interval, they spent a half minute on the platform.

Shuttle box

The passive avoidance apparatus (Shuttle Box) includes bright and dark boxes of a similar size (20×20×30 (cm)) partitioned via a guillotine door (7.9 cm^2). The walls and floor of one box are composed of opaque white resin, while in another box, the walls are dark with a floor covered by electrified bars. The dark compartment's floor is covered by stainless steel electrified bars, to which alternative electric foot shocks (50 Hz, 3s, severe 0.5mA) are applied by a stimulator. Before the experiments, all rats were permitted to maintain in the experimental room for around 30 min. Then, each rat was placed in the apparatus for 5 min. After half an hour, the rats were gradually placed in the white compartment, and after 10s, the guillotine door was opened and the animals entered the dark place (Moshfegh, Babaei, Oryan, Soltani, & Zarrindast, 2011). The rats were excluded when they waited for 2 min to enter the dark chamber. As soon as the rat entered the next box using all paws, the guillotine door was closed, followed by a quick shock. Twenty seconds later, they were removed from the shuttle box and temporarily returned to their home cage. One day later, we placed each rat in the lightroom followed by opening the door after 10 s, then step-through latency (STL) (latency in entering the dark box) was noted as an indicator of inhibitory avoidance response without electric shocks.

Immunohistochemistry

In the first, the samples were deeply anesthetized (Somnotol; 60 mg/kg) (Laferriere & Pang, 2020). The animals were transcardially perfused using ice-cold phosphate buffer saline (PBS) and ice-cold paraformaldehyde (4%) in phosphate buffer (PB) (pH=7.4; 0.1 M), then the brains were immediately removed. Their hippocampus was removed and postfixed in the fixative (paraformaldehyde) overnight (for 24 h) (Gage, Kipke, & Shain, 2012). The hippocampus was mounted and the parts were sectioned transversally on the microtome at 30mm. The slices were kept in PBS with 0.1% NaN3 (sodium aside). Then, to remove any sodium aside from samples, the sections were washed using PBS and then, incubated with the primary antibody. Its primary antibody (Caspase3: ab44976; ki67: ab15580; GFAP: ab7260) was diluted in 0.3% Triton-X (ratio=1:200) in PBS, and then labeled sections were incubated (2 days at 4°C) with gentle shaking. Then each section was washed three times (5 min) in PBS and incubated for 120 min at room temperature using fluorescent conjugate secondary antibodies (rabbit fitc: ab6717). Then the sections were rinsed and mounted and washed again following the secondary antibody incubation. They were observed under a wet mount and post-fixed for 15 min in paraformaldehyde (8%) in 0.1 M PB (pH=7.4), which was crucial to protect signals from hairy cell leukemia (HCL) treatment. In the next step, each section was washed (5 min) three times. Visualization of the GFAP immunoreactivity was done using brown staining with diaminobenzidine (DAB) and H_2O_2 as a substrate within 5 min followed by observation via a light microscope. The optical dissector method was applied as a stereological counting approach using the confocal microscope to determine the rate of positively labeled cells. The all-section results were pooled with a mean of n=1.

Images

The Nikon fluorescence microscope (Optiphot-2) was used to take representative images and an immunoreactive cell was performed at 40X magnification. For quantification, the fluorescent signals were found by a confocal microscope. A single-wavelength laser (ArKr laser; 488 nm) using an LP515 filter was employed to quantify the number of positive cells. The fluorescence images were false-colored and merged using the Adobe Photoshop program (version 7).

Statistical analysis

Data analysis was done by SPSS software version 19 and the Mean±SEM was presented. Variances were analyzed by 1-way analysis of variance (ANOVA) and Tukey's post hoc test was used for multiple comparisons. P values smaller than 0.05 were regarded as significant.

3. Results

Effect of berberine hydrochloride administration on spatial memory and learning using morris water maze (MWM) task

The obtained results from MWM showed that berberine hydrochloride decreases the distance moved and time spent to reach the hidden platform in the four-day learning trails phase and spent time and frequency of motion in the target quadrant on probe test day between groups. The Berberine treatment group indicated a significant reduction ($P<0.01$) regarding distance moved and the time spent to reach the hidden platform in training days concerning the control and addicted groups (Figure 2). We also found significant differences in the distance moved, spent time and frequency of motion in the target quadrant, total distance moved on probe test day between three groups in MWM. As shown in Figure 2, berberine-treated rats spent more time in the target quadrant (zone 1) on the probe test day compared to the addicted group in MWM. Also, the control, addicted and berberine-treated groups indicated a significant difference ($P<0.05$). Based on the ANOVA results, the berberine-treated group had a significant increase in the distance moved in the target quadrant (zone 1) compared to the control and addicted groups on the probe test in MWM. The control, addicted and berberine-treated groups indicated a significant difference ($P<0.05$).

Effect of berberine hydrochloride administration on passive avoidance learning

According to the findings, using berberine hydrochloride decreased the STL in the berberine-treated group compared to the control group ($P<0.05$). The treated and control groups ($P<0.05$), addicted and control groups ($P<0.01$) showed a significant difference in the STL (Figure 3). It is concluded that the addicted group had significant memory impairment compared to the control and berberine-treated groups.

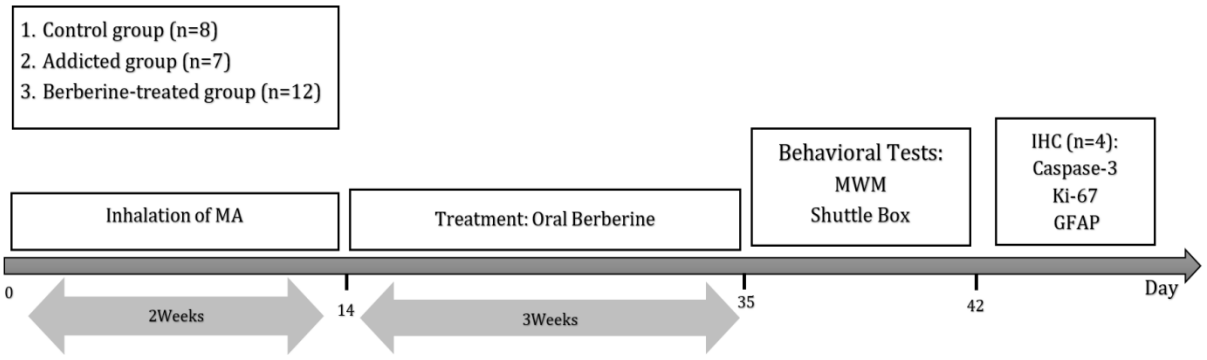

Figure 1. Timeline of experimental procedures, behavioral tests, and outcome measures

MA: methamphetamine; MWM: morris water maze; IHC: immunohistochemistry; GFAP: glial fibrillary acidic protein.

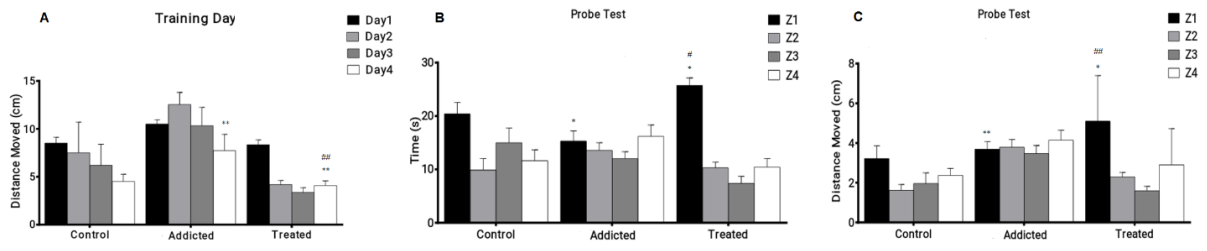

Figure 2. Distance moved (A) of the three groups of rats in the four-day learning trails phase to find the hidden platform in Morris Water Maze (MWM)

The spent time (B) and the distance moved (C) in the target quadrant (zone 1) on the probe test day in three groups in Morris Water Maze (MWM).

A significant difference was observed between control (n=8), addicted (n=7), and berberine-treated (n=12) groups.

(*P<0.05, **P<0.01 vs the control group and #P<0.05, ##P<0.01 vs the addicted group, Mean±SEM).

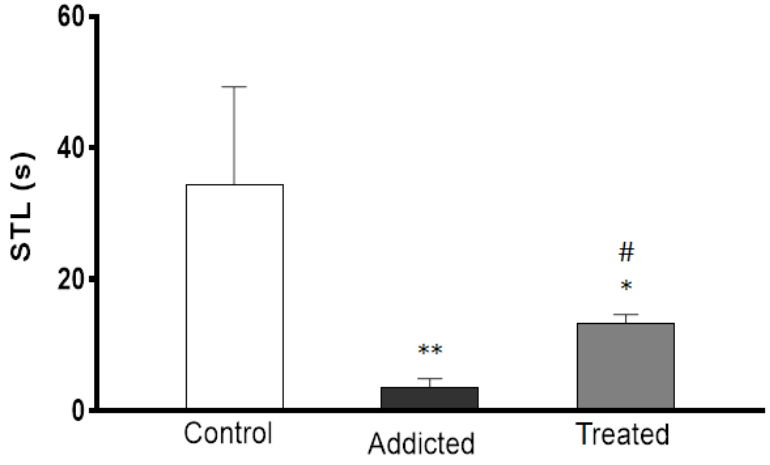

Figure 3. The latency to enter the dark compartment in three groups

A significant difference was observed between control (n=8), addicted (n=7), and berberine-treated (n=12) groups.

(*P<0.05, **P<0.01 vs the control group and #P<0.05 vs the addicted group, Mean±SEM).

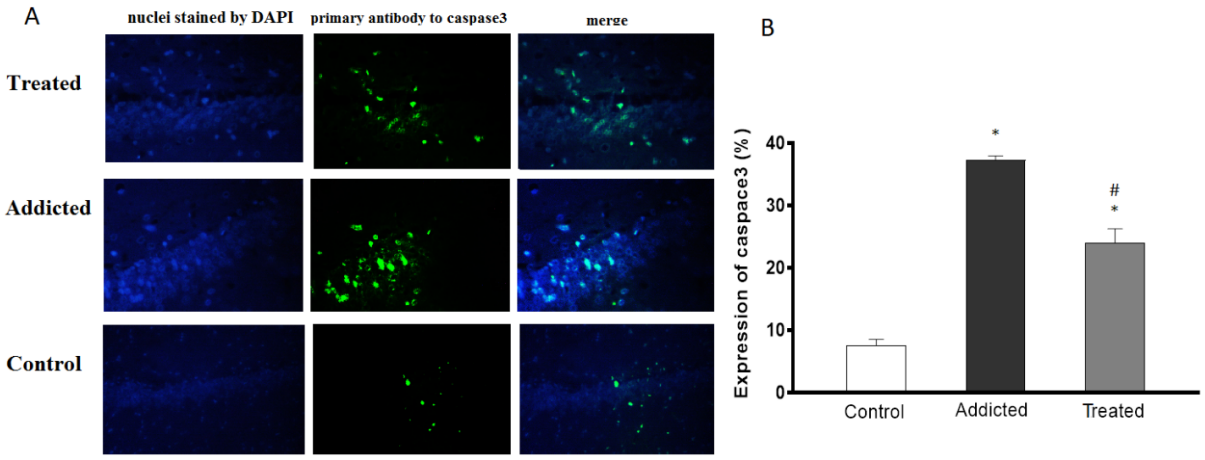

Figure 4. (A) Caspases-3 immunofluorescence staining in three groups (magnification: 400×) NEUROSCIENCE

The antibody in detecting caspases-3 and positive staining is clear (green is caspases-3 staining and total nuclei stained with 4′,6-diamidino-2-phenylindole [DAPI] are blue) (scale bar: 20 µm).

(B) The Mean±SEM of Caspases-3 Proliferation Marker Expression in Three Groups

A significant difference was observed between control (n=8), addicted (n=7), and berberine-treated (n=12) groups.

(*P<0.05 vsthe control group and #P<0.05 vs the addicted group, (n=4), Mean±SEM).

Immunohistochemistry

Effect of berberine hydrochloride administration on caspases-3

In this study, we compared the expression of caspases-3 as a marker of apoptosis in tissue sections taken from the hippocampus of three groups of rats, including control, addicted, and berberine-treated groups. The Mean±SD of Caspases-3 was 7.60±0.92, 37.26±0.70, and 24.10±2.20 in control, addicted, and berberine-treated groups, respectively. Analysis of variance indicated a quantitative significant difference in caspases-3 among the treated and control group (P<0.05), the treated and addicted group, and the addicted and control group (Figure 4). Staining of caspases-3 by immunofluorescence technique in three groups of rats also showed a significant reduction in caspases-3 cells in the berberine-treated group compare to addicted group (P<0.05). The anti-

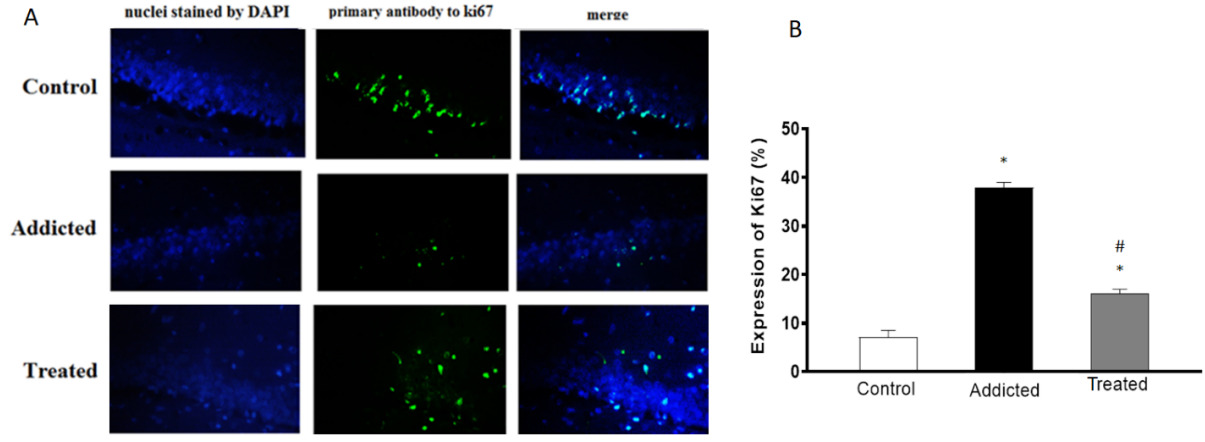

Figure 5. (A) Ki-67 immunofluorescence staining in three groups (magnification: 400×) NEUROSCIENCE

The antibody in detecting Ki-67 and positive staining is clear (green is Ki-67 staining and total nuclei stained with 4′,6-diamidino-2-phenylindole [DAPI] are blue) (scale bar: 20 µm).

(B) The Mean±SE of Ki-67 Proliferation Marker Expression in Three Groups

A significant difference was observed between control (n=8), addicted (n=7), and berberine-treated (n=12) groups.

(*P<0.05 vs the control group and #P<0.05 vs the addicted group, (n=4), Mean±SEM).

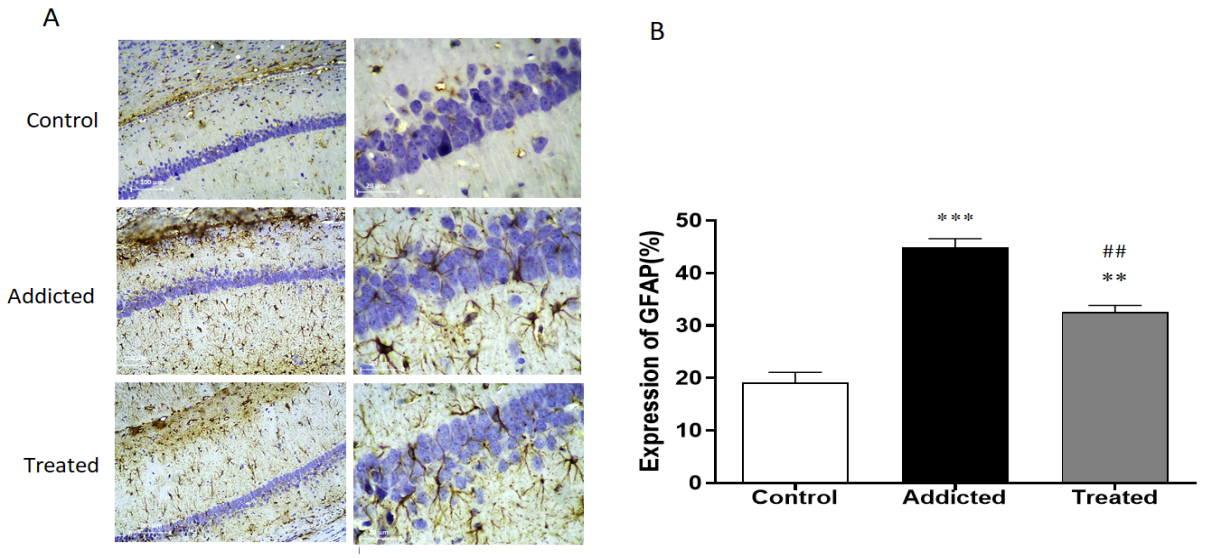

Figure 6. (A) Glial fibrillary acidic protein (GFAP) immunohistochemistry staining in three groups NEUROSCIENCE

The antibody in detecting GFAP and blue is GFAP staining. (Total nuclei stained with DAB are blue, scale bar: 100 µm & 20 µm).

(B) The Mean±SE of Glial Fibrillary Acidic Protein (GFAP) Proliferation Marker Expression in Three Groups

A significant difference was observed between control, addicted, and berberine-treated groups.

(**P<0.01, ***P<0.001 vs the control group and ##P<0.01 vs the addicted group, (n=4), Mean±SEM).

body in detecting caspases-3 in Figure 4A and positive staining (green color) are clear.

Effect of berberine hydrochloride administration on Ki-67

To assess the ongoing proliferation levels in hippocampus tissue, the Ki-67 expression was measured, a nuclear antigen available in cycling cells. The Addicted group indicated a significantly higher Ki-67 expression level compared to the berberine-treated and control groups (P<0.05; Figure 5). Ki-67 Mean±SD was 7.11±1.40, 37.90±1.10, and 16.12±0.90 in control, addicted and berberine-treated groups, respectively. Analysis of variance demonstrated a quantitative significant difference in Ki-67 between the treated and control group (P<0.05), the treated and addicted group, and the addicted and control group (Figure 5). Staining of Ki-67 by immunofluorescence technique in three groups of rats also showed a significant increase in Ki-67 cells in addicted compared to the berberine-treated and control groups (P<0.05). The antibody in detecting Ki-67 in Figure 5, and positive staining (green color) is clear.

Effect of berberine hydrochloride administration on GFAP

GFAP expression is usually applied to assess astrocyte reactivity. Addicted rats indicated a significant increase in GFAP immunohistochemical staining of hippocampus tissue compared to the berberine-treated and control groups. The GFAP Mean±SD was 19.13±3.4, 44.92±2.8, and 32.57±2.2 in control, addicted and berberine-treated groups, respectively. Figure 6 shows the distribution of the GFAP labeling index as measured by the percentage of positively stained cells (DAB staining). Also, it indicated a significant elevation in GFAP expression among the addicted group compared to the berberine-treated and control groups (P<0.05).

4. Discussion

The present research aimed to determine the impacts of berberine hydrochloride administration on cognitive dysfunction caused by MA consumption and brain inflammation was examined by caspase-3, ki-67, and GFAP expression. Based on the findings, berberine led to significant spatial learning and memory improvement in MWM and passive avoidance tasks, significantly decreased activation of caspases-3, higher percentages of Ki67 expression, and increased GFAP expression of cells were found due to MA consumption. Our results show that berberine may have a potential role in the treatment of cognitive impairments induced by MA consumption. Our findings are consistent with the recent studies using animal models that have indicated the beneficial neuroprotective effects of berberine against various disorders of the central nervous system, including Alzheimer, addiction, anxiety, forebrain ischemia, and mental depression.

Our results indicated that berberine decreased caspase-3 (a major component of the apoptotic process) activation in the berberine-treated group more than in the addicted group. Berberine can pass the blood-brain barrier and transport it to neurons depending on concentration and time (G. K. Wang, Edrich, & Wang, 2006). In a study by Lin, J. P., Yang, Lee, Hsieh, & Chung, (2006). apoptosis due to berberine was linked to increased Ca^{2+} levels as well as a reduced mitochondrial membrane potential (MMP) causing cytochrome c secretion and the pro-caspase-3 cleavage. It can increase Bax and cytochrome c amounts and reduce Bcl-2 levels. Conversely, caspase-3 activating inhibition (z-VAD-fmk as a cell-permeable broad-spectrum caspase inhibitor) entirely stopped apoptosis by berberine in human leukemia HL-60 cells as well as mouse leukemia WEHI-3 cells. Therefore, berberine can induce apoptosis in these cells by activating caspase-3 Lin, Kao, Chen, Ho, & Chung, (2006). They found that it can induce p53 expression resulting in a reduction in the MMP, cytochrome C secretion, and caspase-3 activation to induce apoptosis. On the other hand, it decreases the intracellular reactive oxygen species (ROS), however, it is not rare. Treatment with berberine up to 48h and 72h slightly increases cells' viability. Berberine can arrest cells in the S- and G2/M phases of the cell cycle; however, regarding the S-phase, it is transient and dose-dependent, whereas G2/M arrest is more evident (J.-P. Lin, Yang, Lee, Hsieh, & Chung, 2006). One explanation is that the effect of berberine on cancer cells may differ from its effect on normal cells. The transformation of a cell from normal to cancerous in turn may affect not only cell functions but also the cell cycle. Cancer cells have more genetic changes compared to normal cells. Berberine interacts directly with nucleic acids. It also can affect cancer cell cycle progression, leading to cell division impairment (Tillhon et al., 2012).

Withdrawal from MA can enhance proliferation as well as the survival of the newborn progenitor cells in the hippocampus and increased progenitor cells survival is possibly associated with elevated neurogenesis through protracted withdrawal following MA self-administration (Deschaux et al., 2014; Noonan, Choi, Self, & Eisch, 2008; Recinto et al., 2012). A study found that berberine may induce the differentiation of the adult rat mesenchymal stem cell into neurons *in vitro* (40). In contrast with our hypothesis, Ki-67 as a proliferating marker was significantly enhanced in MA addicted group compared to the control and berberine-treated groups. Similarly, according to the findings by Kim et al. (2019), a disturbed number of Ki-67 progenitors with putative glial phenotypes in the medial prefrontal cortex (mPFC) related to MA is possibly more associated with the MA daily dosage and self-administration method, as well. Massanella et al. (2015) found that meth consumers show higher cell proliferation and exhaustion related to immune response (Massanella et al., 2015).

The present findings reveal that our results support recent studies indicating the increased GFAP expression in the MA-addicted group and decreased post-treatment with Berberine. Several studies have documented microglia (Thomas, Walker, Benjamins, Geddes, & Kuhn, 2004) and astrocyte (Guilarte, Nihei, McGlothan, & Howard, 2003) activation in response to MA administration. Evidence indicated that reactive microglia and enhanced GFAP-positive astrocyte density are present in brains exposed to MA (Friend & Keefe, 2013). The glial cells have been shown to have a profound effect on brain performance (Bartzokis, Lu, & Mintz, 2004) and should be considered a potent contributor to cognitive deficits associated with MA (Mandyam, Wee, Eisch, Richardson, & Koob, 2007). Berberine can attenuate neuronal death due to microglial conditioned media. Chen et al. (2014) reported that treatment with berberine 3h post-injury decreased damage to neurons, apoptosis, and inflammation of traumatic brain injury in vivo. It has also been shown to reduce brain damage by reducing the inflammatory mediators' synthesis with glial cells instead of having a direct neuroprotective impact on traumatic brain injury (Chen et al., 2014). Based on the results of Kim et al. (2019), GFAP expression in the hippocampus was increased by inducing ischemia; however, such berberine reduced GFAP expression in the ischemic gerbils. Berberine has a neuroprotective impact on ischemic insult via inhibiting neuronal apoptosis by inhibiting reactive astrogliosis as well as microglia activating.

5. Conclusions

Administration of berberine hydrochloride for 3 weeks improves cognitive function in MA addiction and has the potential of neuroprotective efficacy.

Ethical Considerations

Compliance with ethical guidelines

All experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publication No. 80-23, revised 1996) and were approved by the Research Institute and the local Ethics Committee affiliated with the Shahroud University of Medical Sciences (Registration Code: IR.SHMU.REC.1396.30).

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Authors' contributions

Conceptualization: Leila Rezaeian; Methodology: Hamid Kalalian Moghaddam and Mehdi Khaksari; Investigation: Raheleh Rafaiee, and Leila Rezaeian; Data curation: Raheleh Rafaiee, and Leila Rezaeian; Formal analysis: Raheleh Rafaiee; Writing-original draft: Leila Rezaeian; Writing-review and editing: all authors; Funding acquisition: Leila Rezaeian; Supervision: Hamid Kalalian Moghaddam.

Conflict of interest

The authors declared no conflict of interest.

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