Accepted Manuscript

Accepted Manuscript (Uncorrected Proof)

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

Running Title: Neuroprotective Effects of Berberine

Authors: Leila Rezaeian¹, Mehdi Khaksari², Raheleh Rafaiee³, Hamid Kalalian Moghaddam⁴. *

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.

*Corresponding Author: Email: h.kalalian@shmu.ac.ir

To appear in: Basic and Clinical Neuroscience

Received date: 2020/04/21
Revised date: 2020/09/29
Accepted date: 2020/10/6
This is a “Just Accepted” manuscript, which has been examined by the peer-review process and has been accepted for publication. A “Just Accepted” manuscript is published online shortly after its acceptance, which is prior to technical editing and formatting and author proofing. *Basic and Clinical Neuroscience* provides “Just Accepted” as an optional and free service which allows authors to make their results available to the research community as soon as possible after acceptance. After a manuscript has been technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as a published article. Please note that technical editing may introduce minor changes to the manuscript text and/or graphics which may affect the content, and all legal disclaimers that apply to the journal pertain.

Please cite this article as:

DOI: http://dx.doi.org/10.32598/bcn.2021.1444.2
Abstract

Introduction: Methamphetamine (MA) as an addictive psychostimulant drug affects the central nervous system. The present research aimed at evaluating the impact of Berberine-hydrochloride on cognitive function Improvement and neuroprotective effects in MA Addicted Rats.

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/day) per oral during the three-week period of withdrawal. Two groups received inhaled MA self-administration for two weeks (up to 10 mg/kg). Following the experimental procedures, 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 GFAP expression.

Results: The obtained results from MWM showed that Berberine Hydrochloride decreases (p<0.01) the distance moved and spent time to reach the hidden platform in four-day learning trails phase and there were significant differences in the distance moved, spent time and frequency of motion in target quadrant on probe test day between groups. Berberine Hydrochloride reduced also the latency to enter animals into the dark chamber in the treated group in comparison with the control group (p<0.05). A significant decrease in activation of caspases-3, higher percentages of Ki67 expression and increase in GFAP expression of cells in Addicted group was found to compare with Berberine-treated and control groups (p<0.05).

Conclusions: Berberine Hydrochloride administration for 3 weeks improves cognition function in MA addiction and it has a potential for neuroprotective efficacy.

Keywords: Methamphetamine, Berberine hydrochloride, Cognitive function, Neuroprotective effects, Rat
**Introduction**

Methamphetamine (MA) has known as a strong addictive stimulant that possesses a high addiction liability (Volkow, 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 the MA misuse for a long time has many negative consequences, including addiction, cardiovascular risks, and severe mental disorders. Symptoms of MA psychosis include anxiety, confusion, insomnia, paranoia and visual hallucinations, deterioration of attention, memory loss, and violence that they reflect significant changes in the brain (Winslow, Voorhees, & Pehl, 2007). MA as an addictive psychostimulant drug affects the central nervous system by alterations in the activity of the extracellular monoamine neurotransmitters (dopamine, serotonin, norepinephrine) by promoting their release from nerve terminals. MA increases extracellular dopamine levels through the reverse transport of dopamine 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 of the studies, the acute and chronic methamphetamine users have shown severe functional and structural alterations in brain regions involved in deficits in memory and emotion. Those with MA abuse obtain significantly lower cognitive scores compared with the controls with no drug abuse (Scott et al., 2007).

The study on the long-term effect of MA exposure on cognitive function while testing for object recognition memory, showed cognitive decline function after MA exposure in rats. For example, when one week following MA administration, rats exposure to high MA doses
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, are available to treat MA abuse (Karila et al., 2010). Berberine Hydrochloride is an isoquinoline plant alkaloid, at first gained from Rhizoma Coptidis, 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 decreased pro-inflammatory cytokine synthesis (Lee, Hyun, & Kim, 2010) and decreased the GFAP expression that is a marker for astrocyte activation (Kim et al., 2019) and 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 at evaluating 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.

**Materials and Methods**

**Animals**

The 27 Male Wistar rats (200-250 g) were provided from Pasteur Institute (Tehran, Iran) and were placed in a room (21±3°C) under 12 h light and 12 h darkness. Food and water were freely accessible. The experiments were performed based on the Guideline by the National Institutes of Health for the Care and Use of Laboratory Animals. Studies were performed from 8:00 to 12:00. The samples were then randomly divided into three groups:

1. Control group of 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-week period of withdrawal. Berberine (Sigma-Aldrich; Merck Millipore, Germany) was prepared by dissolve in Salin (Alavijeh, Vaezi, Khaksari, & Hojati, 2019).

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

At the end of the experiment, memory was assessed using MWM and shuttle box and hippocampal sections from 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 for immunofluorescent staining were used (Fig. 1).

**Morris Water Maze test**

Morris water maze (MWM) assesses spatial memory (Asi et al., 2011). MWM included a dark circular pool filling with water (32 cm) at 22±1 °C with 60 cm in high and 150 cm diameter. The circular tank of water was partitioned to four equal quadrants consist of four starting positions, as south, west, north, and east and a 10 cm diameter clear platform stood 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 that was mounted directly above the center of the MWM pool for recording 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 through 4 consecutive days at nearly the same time and they received 4 tests per day and their memory was tested on the 5th day, during which the platform was removed (Probe test). In the interval, they spent half-minute on the platform.

**Shuttle box**

The passive avoidance apparatus (Shuttle Box) includes both bright and dark boxes with a similar size (20 × 20 × 30 cm) partitioned via a guillotine door (7.9 cm²). The walls and floor
of one box is composed of white opaque resin, whereas 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, 3 seconds, Severe 0.5 mA) are applied by a stimulator. Before the experiments, all rats were permitted to maintain in the experimental room for around 30 minutes. Then, each rat was located in the apparatus for 5 minutes. After half an hour, the rats were located in the white compartment gradually followed by opening the guillotine door after 10 seconds 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 using all paws entered the next box then, the guillotine door was closed followed by delivering a shock quickly. Twenty seconds later, they were removed from the shuttle box and returned to their home cage temporarily. One day later, we placed each rat in the lightroom followed by opening the door after 10 seconds then, STL (the latency in entering the dark box) was noted as an inhibitory avoidance response indicator in the absence of 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 PB (pH= 7.4; 0.1 M), followed by removing the brains immediately. 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 30 mm. The slices were kept in PBS with 0.1% NaN3 (sodium aside). Then to remove any sodium aside
from samples the sections, using PBS, were washed followed by incubation with primary antibody. It 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 shaking gently. Then each section was washed three times (5 min) in PBS and incubated for 120 min at room temperature using secondary antibody fluorescent conjugated (rabbit fitc: ab6717). Then the sections were rinsed and mounted followed by washing 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 the signals from the HCL treatment (Palmer et al., 2000). 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$_2$O$_2$ as a substrate within 5 min followed by observing through a light microscope. The optical dissector method as a stereological counting approach using the confocal microscope (22) was applied for determining the labeled cell’s rate positively. 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 LP515 filter was employed for quantifying the positive cell numbers. The fluorescence images were false-colored and merged using the Adobe Photoshop program (Version 7).
Statistical Analysis

Data analysis was done by SPSS 19 software and they presented as mean ± S.E.M. Variances were assessed by one-way analysis of variance (ANOVA) and also Tukey's post hoc test was used for multiple comparisons. P values of smaller than 0.05 were regarded as significant.

Results

Effect of Berberine Hydrochloride administration on spatial memory and learning using 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 spent the time to reach the hidden platform in training days with respect to the Control and Addicted groups (Fig. 2). We also found, there were 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 Fig. 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. The Control, Addicted and Berberine-treated groups indicated also 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) in comparison with 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 step-through latency (STL) in the Berberine-treated group than 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 (Fig. 3). It is concluded that Addicted group had significant memory impairment than the Control and Berberine-treated groups.

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 include Control, Addicted and Berberine-treated groups. Caspases-3 mean score (% ±SD), 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), Treated and Addicted group, and Addicted and Control group (Fig. 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 than addicted (p< 0.05). The antibody in detecting caspases-3 in Fig. 4A, and positive staining (green color) is clear.

Effect of Berberine Hydrochloride administration on Ki-67

For the assessment of the ongoing proliferation levels in hippocampus tissue, the Ki67 expression was measured, a nuclear antigen available in cycling cells. Addicted group
indicated a significantly higher Ki67 expression level in comparison with the Berberine-treated and control groups (P<0.05, Fig. 5). Ki-67 mean score (% ±SD) 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 among the Treated and Control group (p<0.05), Treated and Addicted group, and Addicted and Control group (Fig. 5). Staining of Ki-67 by immunofluorescence technique in three groups of rats also showed a significant increase in Ki-67 cells in Addicted than the Berberine-treated and control groups (p< 0.05). The antibody in detecting Ki-67 in Fig. 5, and positive staining (green color) is clear.

**Effect of Berberine Hydrochloride administration on GFAP**

GFAP expression is usually applied for assessing astrocyte reactivity. Addicted rats indicated a significant increase in GFAP immunohistochemical staining of hippocampus tissue in comparison with the Berberine-treated and Control groups. GFAP mean score (% ±SD) 19.13±3.4, 44.92±2.8, and 32.57±2.2 in Control, Addicted and Berberine-treated groups, respectively. The distribution of the GFAP labeling index as measured by the percentage of positively stained cells (DAB staining) is shown in Fig. 6 also indicated a significant elevation in GFAP expression among Addicted group than the Berberine-treated and Control groups (p< 0.05).

**Discussion**

The present research aimed at determining 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 due to MA consumption was found. Our results show 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 have indicated the beneficial neuroprotective effects of berberine against various disorders of the central nervous system (15) including Alzheimer (35), addiction (36) anxiety (37), forebrain ischemia (38) and mental depression (15).

Our results indicated that berberine decreased caspase-3 (a major component of the apoptotic process) activation in the Berberine-treated group more than Addicted group. Berberine is able to pass the blood-brain barrier and transport to the neurons in a dependent on the concentration- and time (G. K. Wang, Edrich, & Wang, 2006). In a study of Lin et al. apoptosis due to berberine was linked to an increase in Ca^{2+} levels as well as a reduction in the 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 (C.-C. Lin et al., 2006). Lin et al., (2006) found that it is able to 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 through 48 and 72 h, slightly increases cells’ viability. Berberine can arrest cells in S- and G2/M phases.
of the cell cycle, however regarding 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 berberine effects on cancer cells may differ from its effects on normal cells. The transformation of a cell from normal to cancerous in turn may not only affect 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 is able to affect cancer cell cycle progression, which leads 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 the increased progenitor cells survival is possibly associated with the 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 adult rat mesenchymal stem cell differentiation into neurons in vitro (40). In contrast with our hypothesis, ki67 as a proliferating marker was significantly enhanced in MA addicted group than the control and Berberine treated groups. Similarly, according to the findings by Kim et al. (2014), a disturbed number of Ki-67 progenitors with putative glial phenotypes in the 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 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. There is a growing amount of evidence indicated that reactive microglia and enhanced GFAP-positive astrocyte density in brains with MA exposure (Friend & Keefe, 2013). The glial cells have shown with a profound effect on brain performance (Bartzokis, Lu, & Mintz, 2004) and it should be considered as 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 3 h after 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 a direct neuroprotective impact on traumatic brain injury (Chen et al., 2014). Based on the Kim et al. (2014) results, GFAP expression in the hippocampus elevated through inducing ischemia, however, such berberine reduced GFAP expression in the ischemic gerbils. Berberine has a neuroprotective impact on ischemic insult through the inhibition of the neuronal apoptosis by inhibiting reactive astrogliosis as well as microglia activating.

**Conclusions:** Berberine Hydrochloride administration for 3 weeks improves cognitive function in MA addiction and it has a potential for neuroprotective efficacy.

**Ethical Considerations**

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 and the local Ethics Committee affiliated with the Shahroud
University of Medical Sciences, has approved of this study (Registration code: IR.SHMU.REC.1396.30).

**Funding**

This work as a PhD dissertation (Project No. 9648) was supported by Shahroud University of Medical Sciences, Iran.

**Author contributions**

Conceptualization, Leila Rezaeian; Methodology, Hamid Kalalian Moghaddam and Mehdi Khaksari; Investigation, Raheleh Rafeiie and Leila Rezaeian; Data Curation, Raheleh Rafeiie and Leila Rezaeian; Formal Analysis, Raheleh Rafeiie; Writing – Original Draft, Leila Rezaeian; Writing – Review & Editing, all authors; Funding Acquisition, Leila Rezaeian; Supervision, Hamid Kalalian Moghaddam.

**Declaration of interest**

None declared.

**Acknowledgments**

The current research was extracted from a Ph.D. thesis on addiction and the authors gratefully acknowledge to the deputy for research in the Shahroud University of Medical Sciences and those who participated in the study.
References


Fig 1. Timeline of experimental procedures, behavioral tests, and outcome measures. Abbreviations: MA, methamphetamine; MWM: Morris Water Maze; IHC, Immunohistochemistry; GFAP, Glial Fibrillary Acidic Protein.

Fig. 2. The distance moved (A) of the three groups of rats in the four-day learning trails phase to find the hidden platform in 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 MWM. There was a significant difference 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).
Fig. 3. The latency to enter the dark compartment in three groups. There was a significant difference 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).

Fig. 4. (A) Caspases-3 immunofluorescence staining in three groups (magnification: 400×). The antibody in detecting caspases-3, and positive staining is clear (green is caspases-3 staining and total nuclei stained with DAPI are blue) (Scale bar: 20µm). (B) The mean and standard error of caspases-3 proliferation marker expression in three groups. There was a significant difference 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).
Fig. 5. (A) Ki-67 immunofluorescence staining in three groups (magnification: 400×). The antibody in detecting Ki-67, and positive staining is clear (green is Ki-67 staining and total nuclei stained with DAPI are blue) (Scale bar: 20µm). (B) The mean and standard error of Ki-67 proliferation marker expression in three groups. There was a significant difference 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).

Fig. 6. (A) GFAP immunohistochemistry staining in three groups. The antibody in detecting GFAP and blue is GFAP staining. (Total nuclei stained with DAB are blue, Scale bar: 100µm & 20µm). (B) The mean and standard error of GFAP proliferation marker expression in three groups. There was a significant difference 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). *