RESEARCH PAPER

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Neuroprotective effect of *Achillea millefolium* aqueous extract against oxidative stress and apoptosis induced by chronic morphine in rat hippocampal CA1 neurons

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Chronic opioid abuse can impair the hippocampal region of the brain. This study evaluates the neuroprotective effect of *Achillea millefolium* (Ach) on chronic morphine-induced learning and memory impairment, oxidative stress, and neuronal apoptosis in the CA1 region of the rat hippocampus. Thirty-two male Wistar rat rats were classified into four treatment groups (n=8). Morphine sulfate was administered chronically. The treatment groups were given Ach aqueous extract (100, 250, and 500 mg/kg), orally, each day. After 28 days, the Morris water maze test was performed on all subjects. Caspase-3, Bax, and Bcl-2 proteins expression in the CA1 region of hippocampal tissue was analyzed using the western blot method. Also, malondialdehyde concentration, glutathione peroxidase activity, and superoxide dismutase activity were evaluated. The results indicated that Ach extract can improve spatial learning and memory defects in morphine-treated rats. Ach administration also ameliorated apoptosis and oxidative stress indicator levels in hippocampal CA1 of morphine-treated animals. Based on the present study, Ach improved spatial learning and memory defects, and reduced oxidative stress and apoptosis in the hippocampus CA1 region, in chronic morphine-treated animals.

Key words: morphine, *Achillea millefolium*, neurotoxicity, hippocampus, oxidative stress, apoptosis

INTRODUCTION

Morphine has been used as a powerful analgesic for hundreds of years, however, long-term use of morphine is related to dependence and tolerance (Gao et al., 2007). Numerous memory assessment tasks have shown that chronic administration of opioid agents appears to impair learning and memory processes (Farahmandfar et al., 2015). Similarly, it has been shown that acute pre-training administration of morphine limits memory acquisition in numerous paradigms such as active or passive rejection (Izquierdo, 1979), y-maze discrimination (Castellano, 1975), and operant tasks (Bruins and Colpaert, 1999). Furthermore, according to previous studies, opioids can impact hippocampus-dependent memory in the Morris water maze (MWM) (spatial navigation) task, in which pre-training perennial morphine exposure impaired memory acquisition (Li et al., 2001).

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Chronic opioid exposure may reduce hippocampal long-term potentiation, neurogenesis, and spine density (Miladi-Gorji et al., 2011). Additionally, it may alter hippocampal synaptic transmission and impair spatial memory acquisition (Salmanzadeh et al., 2003). Apoptosis and oxidative stress may serve as a mechanism for opioid-induced neurotoxicity in the central nervous system (Guzman et al., 2006). Moreover, chronic morphine application may cause apoptosis in different brain regions, in rats, including frontal, parietal, occipital, temporal, and hippocampal as well as the spinal cord, while morphine addiction may lead to neurotoxicity in parts of the brain, such as the amygdala and hippocampus (Atici et al., 2004; Rezai et al., 2018). Based on experimental studies, apoptotic proteins, like caspase-3, are associated with morphine-induced apoptosis, which leads to changes in brain function and development (Hu et al., 2002).

Chronic exposure to morphine appeared to increase Bax protein expression; however, it may decrease Bcl-2 protein levels in the hippocampus (Boronat et al., 2001; Shibani et al., 2019; Saffar et al., 2020). In rodents, oxidative stress may impair learning and memory and antioxidant therapy was demonstrated to compensate for these deficiencies (Hassanzadeh et al., 2011; Fatemi et al., 2018). As a medicinal plant that grows worldwide, *Achillea millefolium* (Ach), also known as yarrow, has been utilized for the treatment of illness and injuries for centuries (Vitalini et al., 2011). Ach has been employed to treat pain, wounds, and infectious and gastrointestinal diseases (Akram, 2013). It was also demonstrated that neurodegenerative Parkinson's and Alzheimer's diseases might be prevented or treated by Ach in experimental studies (Ayoobi et al., 2019).

Ach is a source of flavonoids, such as apigenin and luteolin, which are its most significant pharmacologically active compounds; luteolin was found to have a protective role against cognitive dysfunction as well as learning deficits in a rat model of Alzheimer's disease (Ayoobi et al., 2019). Furthermore, apigenin has been shown as effective in treating numerous neurological disorders including Parkinson's, neuralgia, and insomnia disease (Patil et al., 2014). Ach contains multiple bioactive elements, such as flavonoids, which are responsible for approximately all of its anti-inflammatory and antioxidant properties (Ivanov, 1967; Chandler et al., 1982). Considering these pharmacological effects and the fact that the hippocampus has an important effect on emotional behavior under opioid addiction (chronic opioid abuse), the present study has attempted to evaluate for neuroprotective effects of Ach against chronic morphine-induced learning and memory impairment, oxidative stress, and neuronal apoptosis in the CA1 region of the rat hippocampus.

METHODS

Animals

Forty male Wistar rats (200 ± 10 g) were obtained from the Rafsanjan University of Medical Sciences animal house and randomly put in separate cages (50 × 26 × 25 cm) with access to water and food and kept under a light/dark cycle (12 h/12 h) at 24 h. The experimental protocol was confirmed by the Research Committee of Rafsanjan University of Medical Sciences. All of the experimental procedures, including tests, treatments, or other interventions were conducted based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals treatment

In March 2018, Ach plant material was gathered from Isfahan Botany Herbarium (voucher specimen no. 9757). Flowering branches of the plant, after washing and drying, were removed and ground. The powder (2 g) was percolated in 200 ml of distilled water for 24 h. The HPLC results used for quantifying the flavonoids in the extract sample found 1.58 mg/g of apigenin and 0.28 mg/g of luteolin.

The rats were randomly arranged into five treatment groups (8 rodents in each groups) as follows: saline; morphine; and morphine + Ach (100, 250, or 500 mg/kg, respectively). The saline group served as control animals without any intervention. The morphine group received morphine at a dose of 45 mg/kg for 28 days orally). The morphine + Ach groups received morphine at a dose of 45 mg/kg (s.c.) plus Ach (100, 200, or 500 mg/kg) (p.o.) daily for 28 days. Following 28 days, behavioral testing was done in all groups.

Morris water maze (MWM) test

Spatial memory and learning can be assessed by the MWM test. The water maze was a dim round pool (diameter = 150 cm, wall height = 60 cm). The circular pool was filled with 25 ± 1°C water, to a height of 30 cm. To divide the water maze into four identical quadrants, four equidistant starting positions were situated around the edge of the maze. In the middle of the goal quadrant, a white get-away platform (10 × 10 cm) was placed 1 cm beneath the surface of the water. To hide the platform, a measure of milk was added to make the water opaque. The escape platform remained continuously in the same quadrant. Lights, furniture, and wall posters with geometrical figures were used for ex-

tra-maze cues. For five successive days, each rat was given a four-trial training session each day; during the training a rat was gently released from the beginning position into the water, facing the pool wall. In every session, the four beginning positions were chosen randomly. However, the rat could swim for 60 s to search out the platform and then rest for 20 s on it. If the rat did not locate the platform within the allocated time, it would be gently placed on it and allowed to remain for 20 s. The maximum time allowed to search for the platform was 60 s so the escape latency in the latter situation would be recorded as 60 s. After finishing the fourth trial, the rat was gently dried and warmed using a heat lamp before returning it to its home cage. The mean escape latencies were determined for the four block-type trials. The escape platform was not placed in the pool and the rat was permitted to swim freely for 60 seconds during the probe (transfer) trial on the seventh day. To assess memory, the time spent inside the goal quadrant was determined. An automatic video tracking system was used to evaluate the animals locomotion (Ethovision software; version 7.1, Noldus Information Technology, Netherlands).

Tissue preparation and western blot analysis

After completing the MWM test, animals were exposed to CO_2 and sacrificed under deep anesthesia. A glass petri dish filled with ice-cold saline was prepared for the brain, which was quickly taken from the skull and hippocampus tissues isolated. Finally, in ice-cold lysis buffer, the hippocampus CA1 region was dissected and homogenized (with, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, 1% NP-40, 0.1% Na deoxycholate; 2 µg each of the protease inhibitors aprotinin, pepstatin A, leupeptin, and 0.5 µmol/l PMSF, pH 7.4). The lysate was centrifuged for 20 min at 4°C at 14000 rpm. The supernatant was transferred to new tubes as the whole cell fraction. Using the Bradford technique, the protein concentrations were measured and the supernatant samples were stored at -80°C until molecular analysis.

To analyze caspase-1, Bax, and Bcl-2 (biochemical markers of apoptosis) in the CA1 region of hippocampal tissue, the western blot method was utilized. In summary, electrophoresis on a 12.5% polyacrylamide gel isolated the protein samples that had the same amount of protein (40 µg) and they were then transferred to a PVDF membrane electrically. The layers were blocked (overnight, at 4°C) in Tris-buffered saline with Tween 20 (TBS-T) (150 mM NaCl, 20 mM Tris-HCl, 0.1% Tween 20, pH 7.4) with 5% skim milk. At that point, the PVDF membranes were incubated with rabbit polyclonal anti-Bax, monoclonal rabbit anti-caspase-3 (1:1000), and Bcl-2 (1:1000) antibodies at room temperature for three hours. All antibodies were diluted in blocking buffer. The blots were rinsed 3 times with TBST and then incubated with horseradish anti-rabbit peroxidase-conjugated secondary antibody (Abcam, 1:5000) for one hour at room temperature. The enhanced chemiluminescence method was used to detect the signals. ImageJ analysis software was used for band density. Actin (β-Actin) was immunoblotted (1:5000) for as a loading control.

Oxidative stress status measurement

To measure the oxidative stress status in CA1 area of hippocampus tissue, the malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity levels in each experimental group were measured via different commercial assay kits (ZellBio, Germany). The hippocampus homogenates supernatant was assayed in strict accordance with the instructions in the assay kits.

Statistical analysis

The normality distribution of the data was evaluated by the Shapiro-Wilk test. The data were shown as mean ± standard error of the mean. The escape latency data from the MWM test was analyzed using one- or two-way analysis of variance (ANOVA). The post-training probe trial test and swimming speed data from the MWM test was analyzed by one-way ANOVA. The protein values and oxidative stress status results were analyzed by one-way ANOVA. To identify specific differences, Tukey's *post hoc* test was used. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant.

RESULTS

Effects of *Achillea millefolium* on the acquisition phase of spatial memory in morphine-treated animals

The effect of Ach on learning and spatial memory in morphine-treated animals was evaluated using the MWM test. Fig. 1 reveals the escape latency for the five groups. Given that the goal of the test is for the animals to learn to swim directly toward the platform, the latency to find the hidden platform gradually reduced during the four days of training in all groups (repeated measure ANOVA, $F_{(3,105)}=231.7$; $p=0.000$). Similar results were found for distance traveled (data not shown).

Analysis of the interaction between groups and days revealed significant differences (repeated measure ANOVA, $F_{(12,105)}$=2.4; p=0.007). *Post hoc* analysis adjustment for multiple comparisons revealed that escape latency was significantly longer on the 4th day in the morphine-treated rats compared to the saline group (p<0.001), indicating poor memory and learning performance as a result of receiving morphine. Treating morphine-addicted animals with Ach (500 mg/kg) shortened the escape latency compared to the morphine-treated rats on the 4th day (p=0.001). These results show that Ach improved morphine-induced memory impairment.

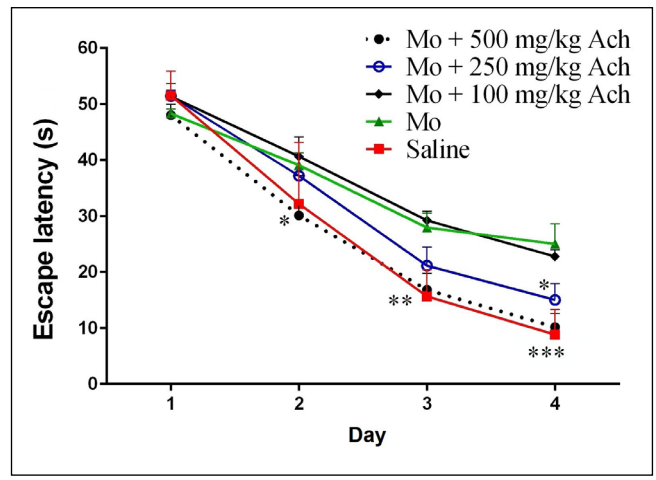

Fig. 1. The effect of *Achillea millefolium* treatment on spatial learning in morphine-treated rats. Each line represents the average escape latency and distance traveled to find the hidden platform in the MWM test for 4 consecutive trial days. Each value is the mean ± SEM. n=8/group. *p<0.05, **p<0.01 and ***p<0.001 *vs.* morphine group at the same time. MO: morphine, Ach: *Achillea millefolium*.

The effects of *Achillea millefolium* on the post-training probe trial test and swimming speed in morphine-treated animals

Based on the probe trial experiment, the rats in all groups similarly searched the maze. Fig. 2A reveals that morphine-treated rats had a reduced ability to remember the escape platform site; therefore, they spent less time in the target quadrant compared to the saline-treated group (ANOVA, $F_{(7,24)}$=11.7; p=0.000). It indicates that chronic morphine treatment caused serious spatial memory impairment. Moreover, rats treated with Ach (500 mg/kg) spent much more time in the target quadrant compared to the morphine group (p=0.001) (Fig. 2A).

The mean swimming speed score was calculated to evaluate locomotor activity within and between groups (Fig. 2B). This factor did not differ between the groups. The results indicated that, with respect to the morphine-treated animals, locomotor activity did not change.

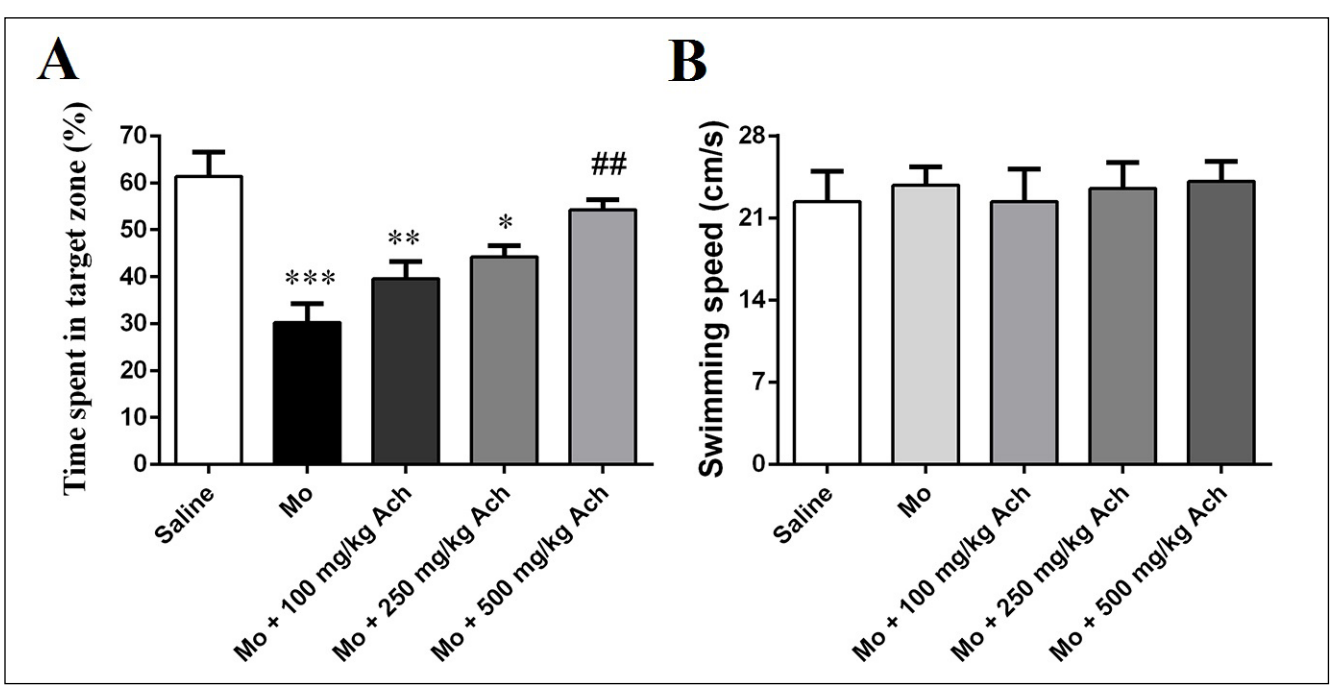

Fig. 2. The effect of *Achillea millefolium* treatment on spatial memory in morphine-treated rats. The percentage of time spent in the target quadrant in the probe task in the MWM test (a). The swimming speed during the probe task in the MWM test (b). Each value is the mean ± SEM. n=8/group. **p<0.01 and ***p<0.001 *vs.* saline (non-morphine–treated) group; #p<0.05 and ##p<0.01 compared with morphine group. MO: morphine, Ach: *Achillea millefolium*.

Effect of *Achillea millefolium* on MDA concentration and SOD and GPx activity in the hippocampal CA1 area of morphine-treated animals

Fig. 3 shows the indicators of oxidative stress and the effects of Ach on lipid peroxidation. One-way ANOVA indicated that malondialdehyde (MDA) levels increased significantly in the morphine group compared to the saline group (ANOVA, $F_{(4,19)}$=12.2; p=0.000). Administration of Ach (500 mg/kg) significantly decreased MDA levels in the chronic morphine-treated animals (p=0.004).

SOD (ANOVA, $F_{(4,19)}$=8.8; p=0.001; Tukey *post hoc* p=0.001) and GPx (ANOVA, $F_{(4,19)}$=7.55; p=0.002) activity decreased in the hippocampus of the morphine-treated animals compared to the saline group (Fig. 3). Moreover, administration of Ach (250 and 500 mg/kg) significantly increased the SOD (p=0.029 and p=0.0041, respectively, Fig. 3) and GPx activity (p=0.023 for Ach 500 mg/kg) levels in the CA1 area of chronic morphine-treated rats.

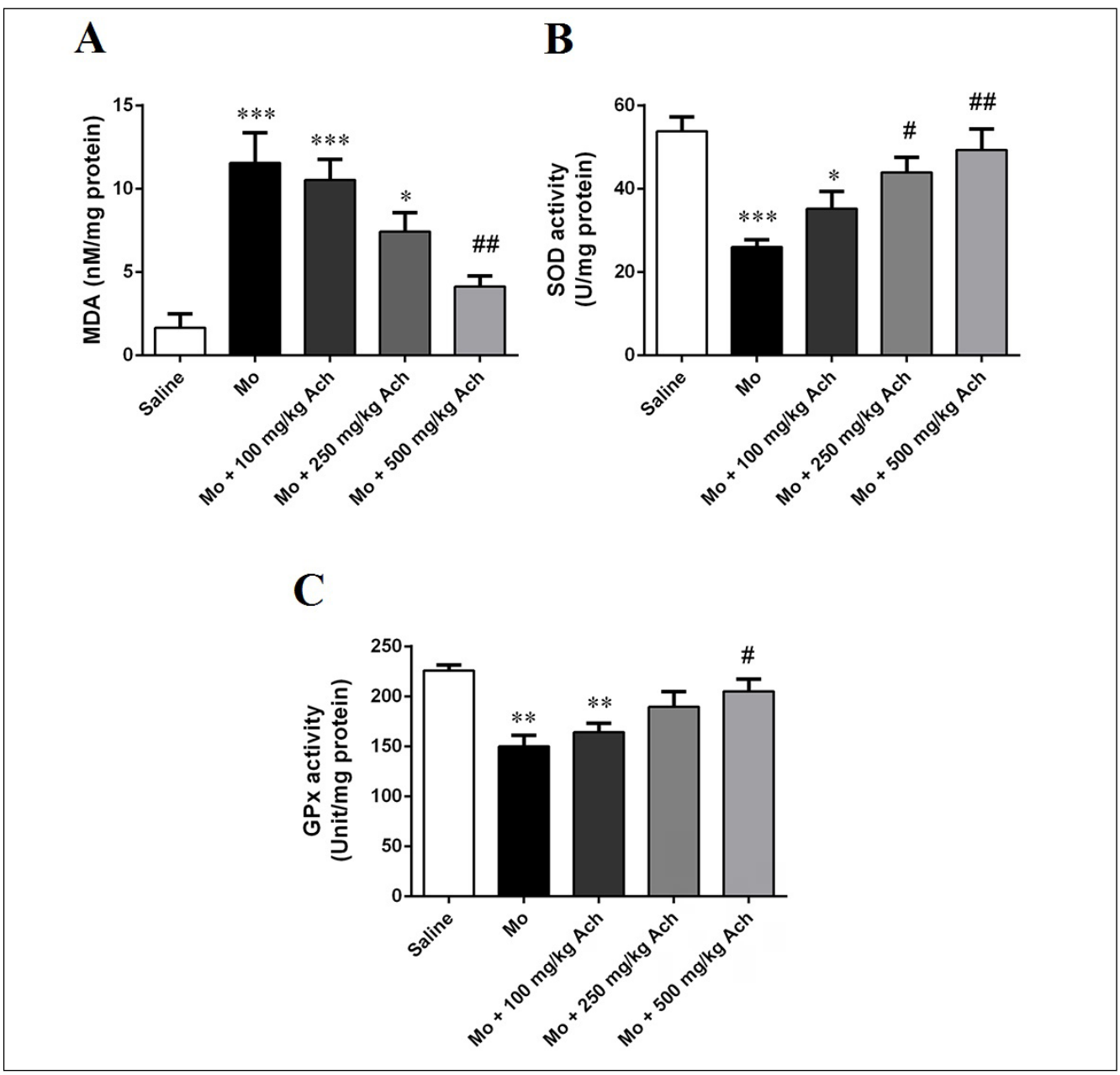

Fig. 3. The effect of *Ach* treatment on lipid peroxidation concentration (a), superoxide dismutase/SOD (b) and glutathione peroxidase/GPx (c) activity levels in the hippocampus of morphine-treated rats. Each value is the mean ± SEM. n=4/group. *p<0.05, **p<0.01 and ***p<0.001 *vs.* saline (non-morphine–treated) group; #p<0.05 and ##p<0.01 compared with morphine group. MDA: Malondialdehyde, MO: morphine, Ach: *Achillea millefolium*.

Effect of *Achillea millefolium* on apoptosis in the CA1 region of morphine-treated animals

The effect of Ach on the expression of Bcl-2 protein as an anti-apoptotic factor and caspase-3 and Bax as pro-apoptotic protein factors, were evaluated using western blot. The analysis indicated that chronic morphine treatment significantly increased cleaved caspase-3 (ANOVA, $F_{(4,19)}$=9.5; p=0.000), Bax (ANOVA, $F_{(4,19)}$=5.8; p=0.005) protein expression, and also decreased the Bcl-2 protein expression (ANOVA, $F_{(4,19)}$=6.2, p=0.004) compared to saline- (non-morphine) treated animals. However, Ach (500 mg/kg) prevented elevation in morphine-induced cleaved caspase-3 (p=0.006) (Fig. 4). In addition, using Ach (500 mg/kg) increased the Bcl-2 protein expression in morphine-treated rats (p=0.025) (Fig. 4). Fig. 4 also shows that 500 mg/kg of Ach significantly reduced the Bax to Bcl-2 ratio in morphine-treated rats (ANOVA, $F_{(4,19)}$=6.8; p=0.002).

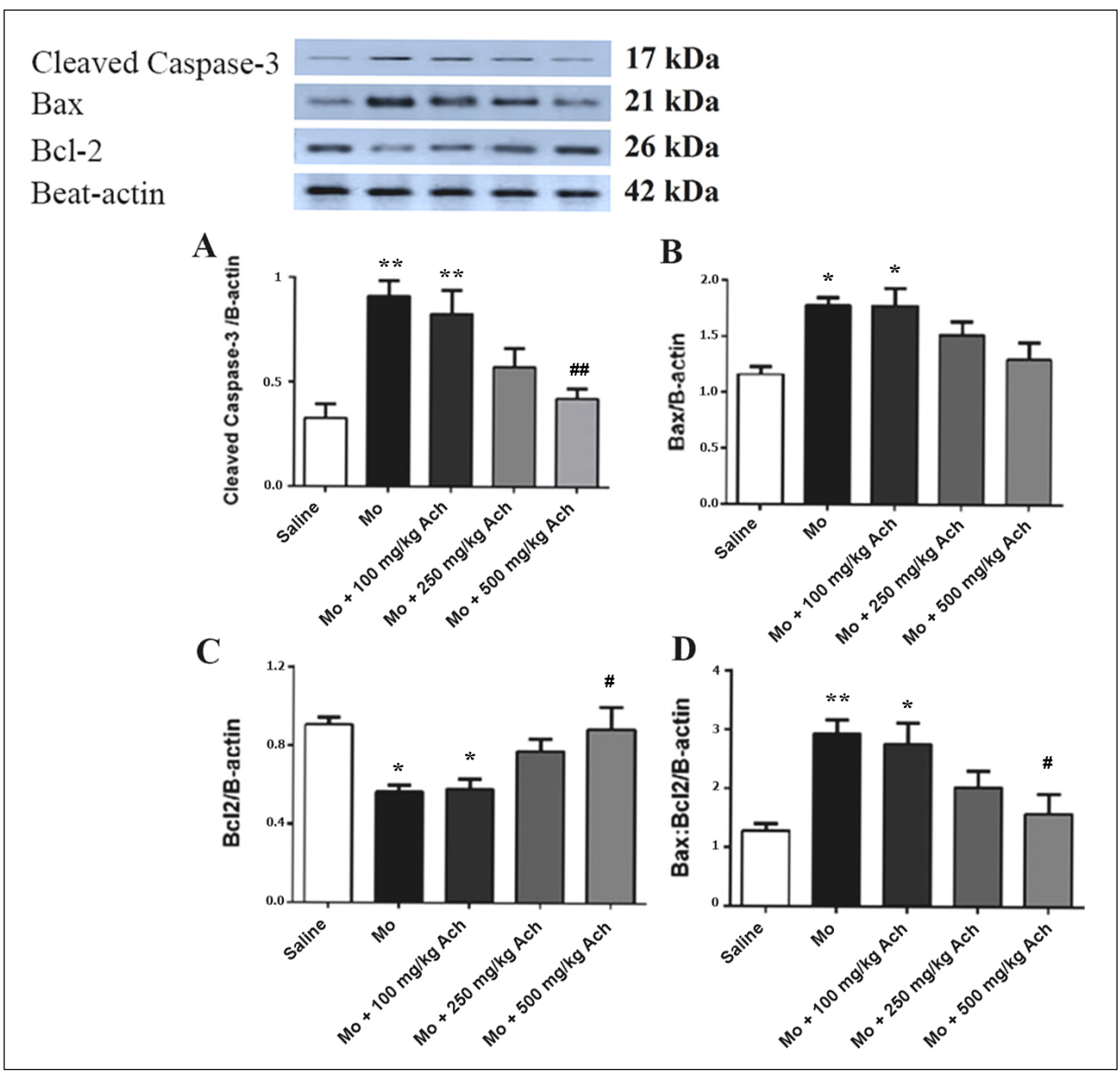

Fig. 4. Western blot analysis of cleaved caspase-3 (a), Bax (b), Bcl-2 (c) protein expression and the Bax/Bcl-2 ratio (d) in the hippocampus of morphine-treated animals. Each value in the graph represents the mean ± SEM of band density ratio for each group. n=4/group. Beta-actin was used as an internal control. *p<0.05 and **p<0.01 *vs.* saline (non-morphine-treated) group. #p<0.05 and ##p<0.01 *vs.* morphine group. MO: morphine, Ach: *Achillea millefolium*.

DISCUSSION

This study explored the protective effects of Ach against neuronal oxidative stress, apoptosis, and cognitive disturbances in the rat hippocampal CA1 region induced by chronic morphine administration. The findings show that chronic morphine treatment significantly impaired cognitive performance, reflected by impaired spatial navigation in the water maze; the morphine-treated rats spent less time in the target quadrant because they could not remember the escape platform site. This result shows that chronic morphine administration induced a spatial memory impairment. In line with our study, it has been shown that morphine administration is involved in various brain structures and can induce memory impairment (Yang et al., 2013; Kaeidi et al., 2015). Moreover, chronic morphine administration impairs spatial learning and causes memory deficits *via* disruption of the hippocampal regions (Lu et al., 2010; Mozafari et al., 2018). However, in line with previous reports, the results of the current study showed that the Ach treatment significantly improved spatial performance. Our results suggest that the Ach can improve morphine-treated memory dysfunction. Ach (500 mg/kg) administration significantly increased the time in target quadrant in morphine-treated rats. In addition, Hendriks et al. (2004) showed that Ach compound administration could suppress behavioral deficits in an experimental autoimmune encephalomyelitis rat model. Also, Jahromi et al. (2019) reported that Ach extract improves the behavioral and memory impairments induced by cerebral ischemia. Thus, our findings and the described studies together demonstrate that Ach is able to improve memory and learning deficits in morphine-treated rats.

The present findings showed that chronic morphine treatment significantly increased MDA levels in the rat hippocampal CA1 area. We also showed that administration of Ach (500 mg/kg) could significantly decrease the MDA concentration in the hippocampus of chronic morphine-treated rats. In line with our study, it has been determined that morphine and other opiates can cause considerable neurotoxicity via the generation of reactive oxygen species and increasing oxidative stress in hippocampus CA1 (Motaghinejad et al., 2015; Shibani et al., 2019). Moreover, our present findings showed that SOD and GPx (two important antioxidant enzymes) activity significantly decreased in the CA1 hippocampal tissue in the morphine-treated rats. Additionally, administration of Ach (500 mg/kg) significantly increased SOD and GPx activity in the hippocampal CA1 area of chronic morphine-treated animals in the present investigation. Meanwhile, Chou et al. (2013) showed that Ach extract decreased serum lipid peroxidation concentration and increased SOD and GPx activity in lipopolysaccharide-induced oxidative stress in RAW 264.7 macrophages. It has also been demonstrated that Ach components exhibit antioxidant activity in different types of disease models (Miguel, 2010). Altogether, this data shows that Ach can inhibit morphine-induced oxidative stress in hippocampal tissue, an important brain structure related to learning and memory functions.

In addition to the other results, immunoblotting analysis in the present study revealed that chronic morphine treatment significantly increased cleaved caspase-3 and Bax protein expression levels and decreased Bcl-2 protein expression, important biochemical biomarkers of apoptosis and cell death in the hippocampus. Accordingly, it was previously reported that chronic morphine administration can increase the expression of pro-apoptotic proteins such as Bax and caspase-3, while decreasing Bcl-2 protein expression and thereby triggering apoptotic mechanisms in hippocampus cells (Liu et al., 2013; Motaghinejad et al., 2015). In the current study, Ach (500 mg/kg) administration was found to ameliorate the morphine-induced cleaved caspase-3 and Bax proteins expression levels in the CA1 area of hippocampus. In addition, Ach (500 mg/kg) administration increased Bcl-2 protein expression in hippocampal CA1 in morphine-treated animals. Zhang et al. (2018) showed that Ach compounds, such as borneol, play the primary role in neuroprotection and prevention of apoptosis in cerebral ischemia, which is consistent with the present study. Therefore, according to the immunoblotting data, it can be claimed that Ach extract has protective effects against morphine-induced cell damage and apoptosis in hippocampal tissue.

CONCLUSION

The current study shows that Ach improves hippocampus-dependent spatial learning and memory and reduces oxidative stress, lipid peroxidation, and apoptosis in the hippocampal CA1 region in chronic morphine-treated rats. In sum, Ach should be considered as a potential beneficial substance for use against chronic morphine administration side effects in brain structures. However, further studies are required to examine the other beneficial effects of Ach in this model.

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