Effects of Combining Methylprednisolone with Magnesium Sulfate on Neuropathic Pain and Functional Recovery Following Spinal Cord Injury in Male Rats

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Received: 24 Sep. 2013; Received in revised form: 30 Mar. 2014; Accepted: 24 May 2014

Abstract- Methylprednisolone (MP) has been widely used as a standard therapeutic agent for the treatment of spinal cord injury (SCI). Because of its controversial useful effects, the combination of MP and other pharmacological agents to enhance neuroprotective effects is desirable. Magnesium sulfate ($MgSO_4$) has been shown to have neuroprotective and antihyperalgesic effects. In the present study, we sought to determine the effect of combining MP and $MgSO_4$, on neuropathic pain and functional recovery following spinal cord injury (SCI) in male rats. A total of 48 adult male rats (weight 300–350 g) were used. After laminectomy, complete SCI was achieved by compression of the spinal cord for one minute with aneurysm clips. Single doses of Magnesium sulfate ($MgSO_4$), (600 mg/kg), Methylprednisolone (MP), (30 mg/kg) or combining $MgSO_4$ and MP were injected intraperitoneally. Prior to surgery and during four weeks of study Tail flick latency (TFL) and BBB (Basso–Beattie–Bresnahan) score and the acetone drop test were evaluated. In mean values of BBB score, a significant difference was observed in SCI+veh compared with other groups (P<0.05). Mean TFL also was significantly higher in SCI+veh compared with other groups (P<0.05). Mean acetone drop test score and weight were significantly different in $MgSO_4$, MP and combining $MgSO_4$ and MP treated groups compared with SCI+veh group (P<0.05). These findings revealed that MP, $MgSO_4$ and combining $MgSO_4$ and MP treatment can attenuate neuropathic pains following SCI in rats include: thermal hyperalgesia and cold allodynia. They also can yield better improvement in motor function and decrease weight loss after SCI in rats compared with the control group.

Acta Medica Iranica, 2015;53(3):149-157.

Keywords: Spinal cord injury; Hyperalgesia; Tail flick; Neuropathic pain; Magnesium sulfate; Methylprednisolone; Cold allodynia

Introduction

Spinal cord injury has profound effects on the body. Despite a variety of treatments SCI, causes rigorous pain in about 40% of patients that is persistent (1). There are many findings of SCI pain mechanisms from experimental models and clinical studies. However, treatment remains hard and inadequate (2). Although huge advances in pharmacotherapy in spinal cord injury (SCI) have been achieved, only MP is used extensively (3). Systemic and intrathecal corticosteroid treatments have been clinically administered with different efficacies in patients with intractable pain (4-6). Also, studies showed corticosteroid therapy can be useful in the prevention and treatment of neuropathic pain (7). Kingery *et al*., showed continuous systemic MP attenuated neuropathic hyperalgesia in sciatic nerve-transected rats (8). A clinical study also showed intrathecal MP relieved postherpetic neuralgia (6). MP, however, has been correlated with increases in side effects; for this reason, its use in treating SCI and its complications is controversial. Thus, combining MP with other pharmacological agents that can promote functional recovery is desirable (9,10).

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Magnesium has long been known as an essential cation necessary for the correct function of more than 300 key enzymes involved in energy renovation, lipid and nucleic acid metabolism and protein synthesis (11). Any decline in magnesium concentration following the neurotrauma will decrease the cells ability to preserve membrane potential (11). It is reported that after head injuries in human, total serum and ionized magnesium concentrations decline (12), which is a dangerous factor leading to permanent tissue damage after direct or indirect neurotrauma (11). Moreover, magnesium supplementation improves treatment outcomes if given before, immediately after, or hours after the injury (13-15). Magnesium deficiency is associated to acute medical/surgical circumstances in which pain or stress exists (16), and leads to hyperalgesia that can be recovered by NMDA antagonists (17).

In current study, we hypothesized combining MP therapy with $MgSO_4$ may have antihyperalgesic effects and may enhance functional recovery following SCI over either treatment alone. Then we sought to find out provide the experimental basis for further clinical application.

Materials and Methods

Animals

Studies were performed on 48 adult male rats (weight, 300–350 g at the start of the experiment). They were purchased from Pasteur Institute, Tehran, Iran. Animals were kept in an environment with a temperature of 23±2°C, 50±5% humidity and a 12-hour light/dark cycle (light: 8.00–20.00).They had free access to tap water and a standard pellet chow. They were handled in accordance with criteria indicated by the Guide for the care and use of laboratory animals (NIH US publication 23±86 revised 1985). All studies were performed according to the International Association for the Study of Pain (IASP) guidelines for animal experiments (Zimmermann, 1983) (18). Animal's weights and cases of SCI complications in rats such as autophagy and mortality were considered and recorded.

Animals (n=6-8 in each group) were randomly divided into five groups:

Sham+ veh Group: Only laminectomy without SCI was performed, and animals received 1ml carrier vehicle (normal saline i.p injection) in the period of 30 minutes after surgery. SCI+veh Group: SCI was performed, and animals received 1ml carrier vehicle (normal saline i.p injection) in the period of 30 minutes after surgery.

SCI+Mg Group: Animals received 600 mg/kg of $MgSO_4$ *i.p.* injection (in 1 ml carrier vehicle) in the period of 30 minutes after SCI. SCI+MP Group: Animals received Methyprednisolone (30mg/kg) *i.p.* injection (in 1 ml carrier vehicle) in duration of 30 minutes after SCI.

SCI+MP+Mg group: Animals received Methyprednisolone (30mg/kg) and $MgSO_4$ (600 mg/kg) *i.p.* injection (in 1 ml carrier vehicle) in the period of 30 minutes after SCI.

Surgery and drug administration

Rats were anesthetized by i.p injection of 50 mg/kg of ketamine (Trittau-Germany). The operation site was prepared by shaving the hair over the skin and disinfecting with 10% of povidone iodine. Standard sterile technique was performed throughout the surgery. Rats from each group were randomly selected and handled by a blinded observer. An incision was made over the thoracic spine at T7–T12 level. After the incision of the dermal and subdermal tissues at the midline, paravertebral muscles were dissected bluntly exposing the lamina bilaterally. Complete laminectomies were performed, exposing the spinal cord at T7–T12. SCI was achieved by compression of the spinal cord horizontally and extradurally for one minute with the aneurysm clips in T9 level. The wounds were then closed with 3/0 silk sutures.

In 30 minutes period after the surgery, 1 mL of 600 mg/kg of $MgSO_4$ (suspended in sterile distilled water) provided from Sigma (St. Louis, MO, USA) was administered intraperitoneally (*i.p.*) in $MgSO_4$ treated groups. 1 ml of 30 mg/kg of MP provided from Sigma (St. Louis, MO, USA) was administered intraperitoneally (*i.p.*) in MP treated groups. Postoperative care involved control of body temperature and prophylactic antibiotic administration to prevent infection (70mg/kg Cefazolin for 7 days). SCI rats received manual bladder expression twice daily for 10–14 days until their bladder functions were fully recovered. We sacrificed rats after four weeks for histological studies (to confirm SCI site of spinal cord is correct)(19).

Behavioral tests

Measurement of thermal hyperalgesia

Tail flick latency (TFL) was measured with Tail Flick Analgesia Meter (IITC life science model 33t, USA) prior to the surgery and at days 0, 7, 14, 21, 28. After a 45-min acclimatization period, TFL was measured by exposing the dorsal surface of the animal tail to a radiant heat source, and the time taken for the conscious rats to take out their tail from the noxious

thermal stimulus was recorded. To reach proper baseline intensity, each control animal was given five test trials, and the strength of the stimulus was adjusted so that tail flick latencies would be between 7 to 8 s. We considered the cut-off time of 8s to prevent tail injury. The mean intensity level was then calculated and used in the following tail flick testing (20).

Functional evaluation

The rats were assessed 24 h after trauma and days 0, 3, 5, 7, 14, 21 and 28 by Basso–Beattie–Bresnahan (BBB) scoring system described by Basso *et al*., (21). This scale includes 21 different levels of movements of the hind limbs. Average BBB scores of both legs were checked. Maximum score was 21. Observers performed the evaluations in a blinded manner (22).

Cold allodynia (Acetone drop test)

Following a 45 min acclimatization time The response to cold stimulation was tested by spraying acetone to the plantar surface of the paw (2-3s) from an estimated distance of 2 cm and classified as: 0, no response; 1, startle response without paw withdrawal; 2, brief withdrawal of the paw; 3, prolonged withdrawal (5-30s); 4, prolonged and repetitive withdrawal (30s) pooled with flinching and/ or licking (23). A significant increase in cold scores in response to acetone application was interpreted as cold allodynia (24). Observers tested cold allodynia in a blinded manner.

Statistical analysis

For data analysis, two-way repeated measure analysis of variance (25) was used followed by Tukey's post-test. $P<0.05$ was considered to be significant. All data are expressed as mean ±SEM. SPSS Software version 18 was used for analysis.

Results

There was no significant difference between groups among all variables at the beginning of the study ($P>0.05$). Following SCI surgery, there was a weight loss in all SCI groups. In SCI+veh group, it was more considerable (Figure 1). There was a significant difference between the mean weight of SCI+veh group and mean weight of other animals in weeks 2, 3, 4 ($P<0.05$). In three drugs administered group, SCI+MP group had higher weight compared with others.

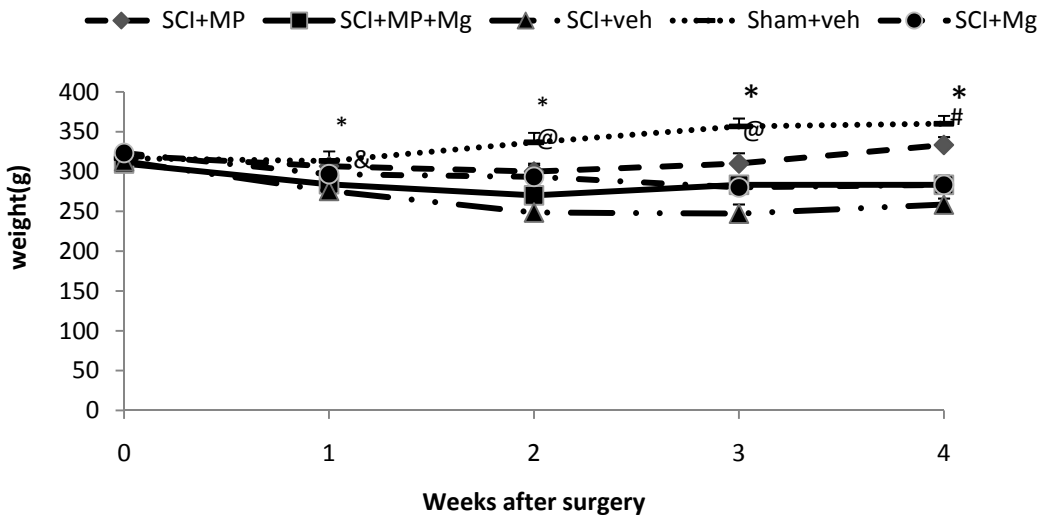

Figure 1. Influence of post-injury administration of $MgSO_4$ and MP on mean weight of rats during four weeks of study
SCI+veh indicate SCI rats received normal saline. SCI+Mg indicate SCI rats treated with $MgSO_4$ (600 mg/kg i.p). SCI+ Mp indicates SCI group treated with MP (30mg/kg). SCI+MP+ Mg indicates group treated with both $MgSO_4$ (600 mg/kg i.p) and MP (30mg/kg). The data are presented as mean ± SEM (6-8 rats per groups). *: $P<0.05$ indicates a significant difference between sham+veh group compared with SCI+MP+Mg, SCI+veh, and SCI+Mg. #: $P<0.05$ indicates a significant difference of SCI+MP compared with SCI+MP+Mg and SCI+veh. @: $P<0.05$ indicates a significant difference between SCI+MP group compared with SCI+veh and sham+veh groups

BBB test

After recovering from anesthesia, animals in the SCI groups displayed a loss of function in their ipsilateral hindlimb. A successful surgery was considered when the animals showed a paralysis of the ipsilateral hindlimb with no simultaneous insufficiency in any of the other

limbs. Animals that showed deficits in other limbs did not recover functionally, or displayed degrees of autophagy were excluded from the study. Over the four weeks period, the rats gradually used their ipsilateral hindlimb. The level of this recovery was measured using the BBB scale. Primarily, the animals exhibited little or no movement in any of the joints of the affected limbs. Motor Functional recovery began with the movement of the hip joint, followed by movement of the knee joint, and at last, movement of the ankle joint. Through the time of motor recovery, the influenced hind limb went through stages of paw curling, abnormal placement, and irregular weight bearing. As it is shown in figure 2, after SCI surgery mean BBB score significantly decreased (P<0.05). In sham+veh group, it did not change significantly. Several measurements in 4 week after surgery showed treatment with $MgSO_4$ (600 mg/kg) and MP (30mg/kg) or combining MP and $MgSO_4$ caused a significant increase in mean BBB score on day 7 compared with SCI+veh group (P<0.05). Also on day 28, a significant difference was observed between the mean score of BBB in SCI+veh group and other groups (P<0.05). In other days of measurement of BBB, there was not any significant difference between mean BBB scores in SCI groups (P>0.05) (Figure 2). Hematuria and the urinary bladder dilation occurred in some of the paraplegic animals and exhibited remission associated with significant improvements in motor function.

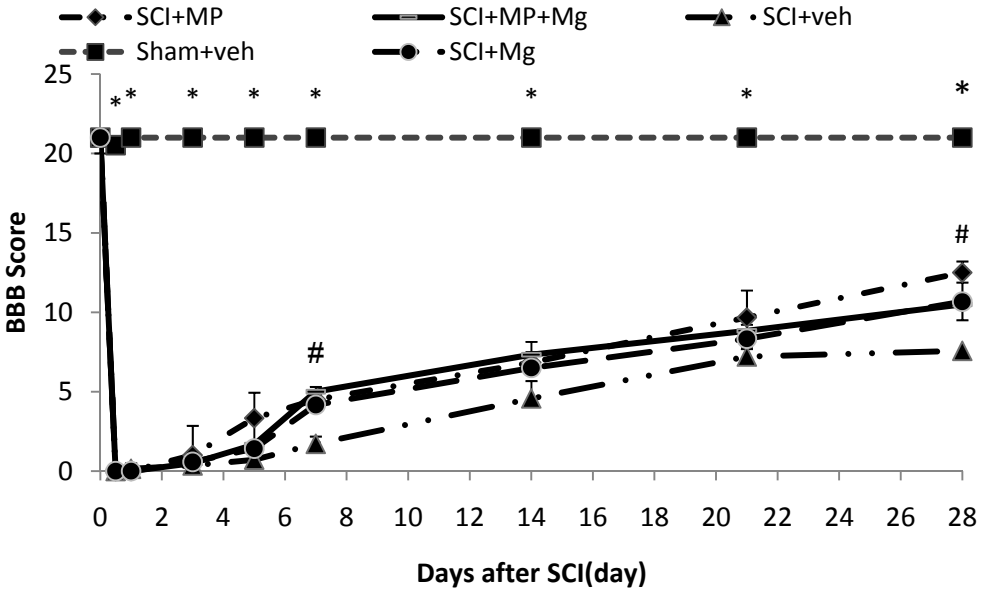

Figure 2. Influence of post-injury administration of $MgSO_4$ and MP on the motor function of rats in four weeks period of study
SCI+veh indicate SCI rats received normal saline. SCI+Mg indicate SCI rats treated with $MgSO_4$ (600 mg/kg i.p). SCI+ Mp indicates SCI group treated with MP (30mg/kg). SCI+MP+ Mg indicates group treated with both $MgSO_4$ (600 mg/kg i.p) and MP (30mg/kg). The data are presented as mean ± SEM (6-8 rats per groups). #: P<0.05 indicates a significant difference between mean BBB score of SCI+veh group compared with other groups. *: P<0.05 indicates a significant difference between mean BBB score of sham+veh group compared with other groups

Tail flick latency

In SCI rats, the reactions to thermal stimuli (tail flick test) showed increased sensitivity relative to pre-surgical tests that were statistically significant (P<0.05) (Figure 3). Mean tail flick latency in SCI groups significantly reduced one week after surgery and it remains low until the second week (P<0.05). In week three after surgery, this value showed a significant increase in SCI+Mg, SCI+MP and SCI+MP+Mg groups. In the week four mean TFL in SCI+Mg group was near to sham+veh and mean TFL of SCI+veh were significantly lower than other groups (P<0.05). In SCI+veh group mean TFL decreased from the first week after surgery and continue to decrease for four weeks (Figure 3).

Cold allodynia

In SCI rats, the responses to cold stimuli (acetone drop on the paw) showed significantly increased sensitivity compared to pre-surgical tests (Figure 4) (P<0.05). Mean acetone test score in SCI+veh and SCI+Mg groups increased one week after SCI surgery

and remained high until the second week. Between second and third week, it reduced in SCI+Mg group, but in SCI+veh group it continued to increase. In fourth week, mean acetone test score in SCI+veh group was significantly higher than other groups (P<0.05), though differences between other groups were not statistically significant (P>0.05).

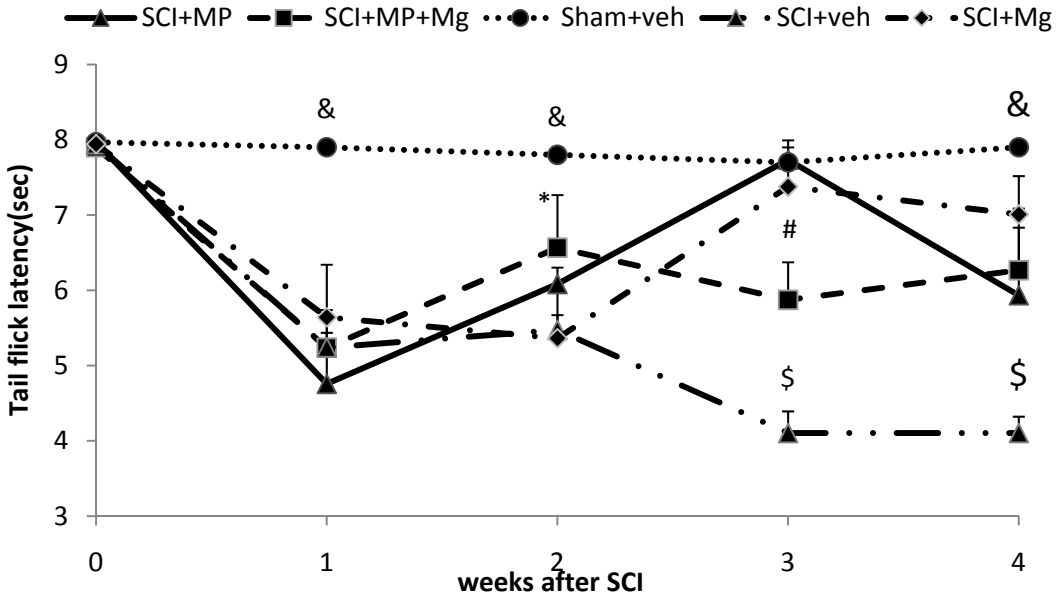

Figure 3. Influence of post-injury administration of $MgSO_4$ and MP on the mean tail flick latencies of rats in four weeks period of study SCI+veh indicate SCI rats received normal saline. SCI+Mg indicate SCI rats treated with $MgSO_4$ (600 mg/kg i.p). SCI+ Mp indicates SCI group treated with MP (30mg/kg). SCI+MP+ Mg indicates group treated with both $MgSO_4$ (600 mg/kg i.p) and MP (30mg/kg). The data are presented as mean± SEM (6-8 rats per groups). #: P<0.05 indicates a significant difference of mean TFL of SCI+MP+Mg group compared with other groups. *: P<0.05 indicates a significant difference between SCI+MP+Mg group compared with SCI+veh and sham+veh groups. $: P<0.05 indicates a significant difference of mean TFL of SCI+veh group and the other groups. &: P<0.05 indicates a significant difference of mean TFL of sham+veh group and the other groups

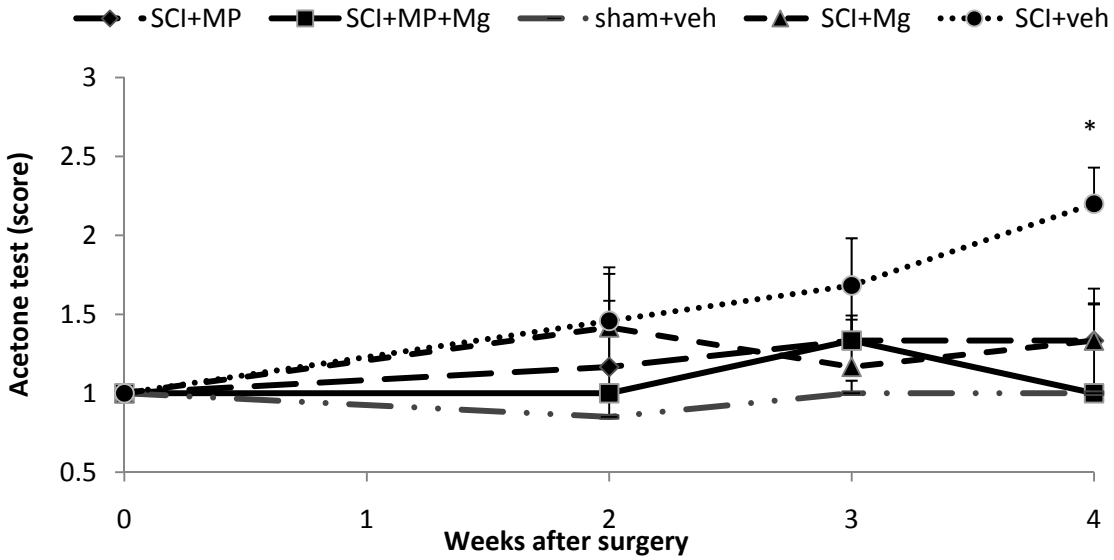

Figure 4. Influence of post- SCI administration of $MgSO_4$ and MP on the acetone test scores of rats in four weeks period of study. SCI+veh indicate SCI rats received normal saline. SCI+Mg indicate SCI rats treated with $MgSO_4$ (600 mg/kg i.p). SCI+ Mp indicates SCI group treated with MP (30mg/kg). SCI+MP+ Mg indicates group treated with both $MgSO_4$ (600 mg/kg i.p) and MP (30mg/kg). The data are presented as mean ± SEM (6-8 rats per groups). *: P<0.05 indicates a significant difference between mean acetone score of SCI+veh and other groups

Discussion

In the present study, single *i.p.* injection of $MgSO_4$, MP and combining $MgSO_4$ and MP within 30 minutes period after SCI, caused better improvement of motor function and attenuating hyperalgesia and allodynia in four-week period after SCI surgery. Several studies have shown SCI leads to chronic neuropathic pain (1,2,26). Current data also showed neuropathic pain symptoms after SCI that was exhibited with lower thermal pain threshold that means thermal hyperalgesia and a higher score in acetone drop test that means cold allodynia.

MP is used in patients with SCI, to reduce neurological injuries, whereas its administration in traumatic SCI within the last few years raises a lot of controversy, and the side effects of its application may be more important than the potential benefits (27). Studies have shown analgesic effects of the corticosteroids. Corticosteroid therapy can be helpful in the prevention and treatment of neuropathic pain (7). Kingery *et al*., showed constant systemic MP attenuated neuropathic hyperalgesia in sciatic nerve-transected rats (8). A clinical study also showed intrathecal MP reduced postherpetic neuralgia (6). Considering MP has many side effects; studies have suggested combining MP with other pharmacological agents may be helpful (9,10).

Magnesium has long been known as an essential cation necessary for the correct function of over 300 key enzymes (11). $MgSO_4$ is an important neuroprotective agent in experimental neurodegeneration and central nervous system damages (13,28-31). It has been shown $MgSO_4$ can improve neurological function of SCI rats compared with controls (19). The decline in tissue magnesium level is an essential pathophysiological factor in secondary SCI (32) and using $MgSO_4$ depresses apoptotic cell death after SCI (33). So in this study, we selected $MgSO_4$ as a neuroprotective agent that in combination with MP may attenuate neuropathic pain following SCI.

We reported a moderate weight loss in SCI groups in weeks after surgery, which was significantly lower in $MgSO_4$ and MP and combing Mp and $MgSO_4$ treated groups compared with SCI+veh group. This reduction is supposed to be due to the pain and disability after SCI and difficulty in food and water accessibility. Between drug administered groups, weight of MP treated group was higher than others. Combination of MP and $MgSO_4$ did not lead to a significant difference in rat's weight compared with MP or $MgSO_4$ treated groups. Generally we can suppose better neurological and functional circumstances in rats treated with Mg and MP led to higher weight in these groups. Wiseman *et al*., did not report weight loss in SCI rats after surgery in control or $MgSO_4$ treated groups (19).

In present study, SCI rats received MP, $MgSO_4$ or combining MP and $MgSO_4$ had significantly higher BBB scores compared to SCI+veh rats that mean more improvement in motor function. However, Combining MP and $MgSO_4$ did not lead to higher BBB score compared with using them alone. Kaptanoglu *et al*., (34) showed significantly higher BBB score in $MgSO_4$ treated SCI rats compared with the control group. Kohno *et al*., (35) in a study of magnesium prophylaxis for protection against spinal cord ischemia, using the scoring system proposed by Marsala and Yaksh (36), reported a considerable improvement in neurological condition during the early postischemic period in animals treated with magnesium (35). In the case of the effect of MP on BBB score, Yin *et al*., reported no significant difference between MP treated group and control SCI, but combination of MP and rolipram caused a significant higher BBB score compared to control SCI that indicated better functional recovery (9). Chengke *et al*., in a study reported increased BBB score on the 14th and the 21th days after ASCI (acute spinal cord injury) in MP treated SCI rats (37). Also, Ji *et al*., (10) showed a higher BBB score in SCI rats treated with MP compared with control SCI rats. These studies to some extent confirm results of the present study.

Analgesic and antihyperalgesic effects of $MgSO_4$ and MP have been reported in several studies (6, 8, 38-40), but according to our knowledge their effect on neuropathic pain following SCI when administered in combination was not reported previously.It is shown that SCI rats had significantly lower TFLs compared with normal rats (41). It means thermal hyperalgesia. Observing this phenomenon in current study 3-4 weeks after SCI, there was significantly higher mean TFL in SCI groups treated with $MgSO_4$, MP and combining MP and $MgSO_4$ compared with SCI+veh group in four weeks. Higher mean TFL in MP and Mg treated groups indicates lower heat hyperalgesia than that in control SCI. Combining administration of MP and $MgSO_4$ did not lead to significantly different results compared with administration of MP or $MgSO_4$ alone. Rond´on *et al*., showed magnesium attenuates thermal hyperalgesia in a rat model of diabetic neuropathic pain (18). Mert *et al*., reported intraplantar coadministration of fentanyl and magnesium can prevent delayed thermal hyperalgesia in rats (42) Takeda *et al*., showed MP significantly inhibit the development of thermal hyperalgesia in spinal nerve

ligation induced thermal hyperalgesia in rats (7). Johansson *et al.,* in a study on neuropathic pain-like condition following chronic constriction injury to the left sciatic nerve in rats showed the heat hyperalgesia were depressed in the animals receiving the corticosteroid (MP) but not in those treated with saline (43). Takeda *et al.*, in a study in a rat model of spinal nerve ligation reported systemic and intrathecal MP inhibited the increase and maintenance of neuropathic pain (thermal hyperalgesia) condition (44). In contrast, Kingery *et al.,* reported MP administered systemically (3 mg/kg, i.p., daily for three weeks) did not attenuate the heat-hyperalgesia that is seen when saphenous afferents sprout into the denervated territory of the sciatic nerve (45). This controversy may exist because of diversity in the causes of hyperalgesia in those studies.

Consistent with previous studies, that reported allodynia following SCI (46), results of the present study showed cold allodynia in SCI groups. $MgSO_4$, MP and combination of $MgSO_4$ and MP significantly reduced cold allodynia. Combining MP and $MgSO_4$ caused attenuation of cold allodynia but this effect did not show a significant difference compared with their effects when they were administered alone. In a study on oxaliplatin-induced peripheral neuropathy in rats, Sakurai *et al*., reported the pre-administration of calcium or magnesium (0.5 mmol/kg, i.v.) before oxaliplatin or oxalate prevented the cold hyperalgesia (47). In another study on spinal nerve ligated rats that experienced neuropathic pain, Ulugol *et al*., reported $MgSO_4$ exhibited a significant anti-allodynia effect. It reduced cold allodynia in doses of 250mg/kg (24). Hayashi *et al*., showed systemic corticosteroid (Triamcinolone) therapy has no effect on cold allodynia in chronic constriction injury (CCI) model of painful peripheral neuropathy (48). In another study it was reported that daily administration of corticosteroid (dexamethasone 25 or 50 mg/kg, i.p) cause prolonged anti-allodynia effects (cold and thermal hypersensitivity) (49). As literatures showed, findings of the present study were consistent with reports of previous studies.

Limited information is available about the mechanism of antihyperalgesic and pain reducing effects of MP. Christensen *et al*., in a study on patients with CRPS (complex regional pain syndromes) indicated, steroid inhibition of substance P release could explicate the effectiveness of corticosteroids in reducing pain and edema (50). Steroid inhibition of eicosanoid and cytokine synthesis is another probable mechanism for the analgesic effect of steroids (45). Kenji Taked *et al*., speculated that inhibition of the astrocytic activation by MP led to prevention and alleviation of neuropathic pain in their study.

Inhibition of prostaglandin production may also contribute to this process (44). Hayashi *et al*., in a study concluded the effects of glucocorticoid treatment on neuropathic pain are specifically related to mast cells and that mast cells are involved not only in the initiation of neuropathic pain (51,52), but also in its continuation. Mast cells release a range of mediators that can directly or indirectly stimulate nociceptors, including TNFα, and other mediators that play an essential role in the recruitment of leukocytes to the location of the injury (48). One hypothesis on the pathophysiology of neuropathic pain following SCI indicated the role of spinal cord NMDA receptor channels in central sensitization (39). While magnesium is an antagonist of these channels (53), this mechanism may also be implicated in the increase of thermal pain threshold in $MgSO_4$-treated SCI rats (39).

Magnesium exhibits neuroprotective behavior through a number of mechanisms, such as dilatation of cerebrovascular arteries, blockage of NMDA receptors, and blockage of voltage-gated calcium channels. In addition, directly inhibiting lipid peroxidation and preventing depletion of glutathione, this element may decrease the severity of endothelial and neuronal reperfusion injury (54-56). In this regard, Beril Gok *et al*., showed $MgSO_4$ treatment instantly after SCI prevents neutrophil infiltration after contusion injury to the spinal cord by attenuating chemotaxis (29). Moreover, magnesium may cause vasodilatation of spinal cord vessels by stimulation of endothelial prostacyclin release (34), inhibiting lipid peroxidation (54), or prevent thrombosis of critical segmental vessels by inhibiting platelet reactivity (22).

In conclusion, despite limitations of the current study present findings showed $MgSO_4$, MP and combination of $MgSO_4$ and MP can attenuate thermal hyperalgesia and cold allodynia after SCI. It also appears to improve motor function and reduce weight loss after SCI. We suggest MP and $MgSO_4$ do not reinforce each other. However further research is needed to explain detailed mechanisms of the effects observed. Potential adverse effects of $MgSO_4$ administration combining with MP should be closely investigated. Another study on mechanical and chemical hyperalgesia is required.

Acknowledgment

The authors thank Professor Ahmad Reza Dehpour and Dr Mehrak Javadi-paydar for their collaborations.

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