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Original Investigation

The Effect of Memantine on Functional Recovery of the Sciatic Nerve Crush Injury in Rats

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ABSTRACT

AIM: Following severe peripheral nerve injury (PNI), regeneration is often insufficient and functional recovery is incomplete. In this regard, glutamate N-methyl-D-aspartate (NMDA) receptor antagonist such as Memantine have been shown to have neuroprotective effects. We evaluated the effects of Memantine against sciatic nerve crush injury in male Wistar Rats.

MATERIAL and METHODS: Memantine or vehicle was given parenterally to rats for 7 days postoperative. In Memantine treatment groups, a single dose of agent (5 and 10 mg/kg) was administered daily. The control group was given vehicle in the same manner. The rats were subjected to crush injury in the left sciatic nerve with non-serrated clamp for 30 seconds. Behavioural, electrophysiological and morphological alterations were evaluated during the experimental period.

RESULTS: Results showed that Memantine has no significant effect on regeneration process rate and functional recovery quality. In the sciatic functional index (SFI) test no significant difference was observed between Memantine treatment groups (5 and 10 mg/kg) at any week.

CONCLUSION: Since the major neuroprotective effect of Memantine is due to its protective activity against NMDA receptor-mediated excitotoxicity, it seems that glutamate excitotoxicity is less important in motor impairment due to sciatic nerve crush injury. It is clear that more research is needed to confirm these findings.

KEYWORDS: Memantine, Sciatic nerve, Motor function, Injury, Regeneration, Peripheral nerve injury, Motoneuron

INTRODUCTION

Peripheral nerves injuries result in loss of motor, sensory and autonomic function of the affected nerves. Spinal neural circuits undergo continuous functional and structural changes in response to a peripheral nerve injury (2). Some recent studies show that glutamate receptors may be involved in sensory and motor neuron apoptosis following severe nerve injury in rats (14,17,25,50). Glutamate is an important excitatory neurotransmitter in the nervous system. Neural trauma triggers massive release of glutamate from injured cells (2). Overactivation of the N-methyl-D-aspartate (NMDA) glutamate receptor subtype can result in a process called excitotoxicity that leads to cell death (20,32). Excitotoxicity is due to excessive calcium ion influx which,

in turn, triggers free radical formation and multiple pathways leading to the initiation of apoptotic-like damage (1,5). Therefore, glutamate excitotoxicity is an attractive therapeutic target for attenuation of neural tissue damage (13,32). Hence, NMDA receptor antagonists could potentially provide neuroprotective effects in several neurodegenerative diseases manifesting excessive stimulation of NMDA receptors, including stroke, nerve injury and neuropathic pain syndromes (2,21). However, attempts to use potent competitive NMDA receptor antagonists as neuroprotectants have shown serious side effects in patients (42).

Memantine is a non-competitive N-methyl-d-aspartate glutamate (NMDA) receptor antagonist and is capable of blocking excitotoxicity (12). This drug only reduces excessive NMDA receptor activation when it is pathologically activated for long



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periods of time under excessive glutamate exposure (12). Many *in vitro* and *in vivo* studies have shown the neuroprotective effects of NMDA receptor antagonists. *In vitro* studies using neuronal cell cultures showed that glutamate excitotoxicity was inhibited by Memantine (46). Also, some studies showed that Memantine attenuated neuronal damage caused by spinal cord and optic nerve ischemia (11,18). Memantine could limit neuronal loss in animal models of stroke and peripheral ganglion injury models (7,43). In addition, Memantine has shown neuroprotective effects against amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) disease models (47). Furthermore, several clinical trials also showed the beneficial effects of Memantine for the treatment of Alzheimer's disease (AD) (31) and Parkinson's disease (PD) (24). Therefore, due to the lack of sufficient information concerning the neuroprotective properties of Memantine on the functional recovery of sciatic nerve injury, this experimental study was conducted to evaluate the probable effect of Memantine on functional recovery of crushed sciatic nerves in Wistar rats.

■ MATERIAL and METHODS

All animal experiments were carried out in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC), and in accordance with the local Ferdowsi University of Mashhad (FUM) committee for Human and Animal ethics.

Memantine and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA) while ketamine and xylazine were obtained from Alfasan Pharmaceutical Co. (Holland) in injectable form. Memantine was dissolved in DMSO. All drugs were injected intraperitoneally (i.p.) and fresh drug solutions were prepared each day of the experiments.

All experiments were performed on adult male Wistar rats (weighing 250–300 g, aged 3 months). Animals were maintained under standardized housing conditions (temperature, $22 \pm 2^\circ\text{C}$, 12-h light/dark cycle light on from 7 a.m. and $60 \pm 5\%$ humidity) in plexiglas cages with free access to food (standard laboratory rodent chow) and tap water *ad libitum*. Experiments were carried out between 9 a.m. and 12 p.m. Ten rats were used for each treatment group.

All experiments were performed under an operating microscope in sterile conditions by the same investigator. All animals were deeply anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The skin was shaved and disinfected using 10% povidone iodine. Then, the rats were fixed in the prone position on the operating table under sterile conditions. The left sciatic nerve was exposed through a longitudinal incision extending from the greater trochanter to the mid-thigh. Then, a 3 mm-long segment of the sciatic nerve was crushed by maximally clamping the nerve with non-serrated hemostatic forceps (Belge de Gembloux, Belgium) for 30 s at a position 1 cm below the sciatic notch. This procedure causes axonal interruption but preserves the connective sheaths (axonomesias). For limiting the inter-animal variability in the postoperative outcome followed by microsurgical neuroorrhaphy, the crush model was

used. The crush model is appropriate to assess the roles of different agents in the nerve regeneration process and for pharmacological investigation (41). All surgeries were done using the same forceps. The nerves were kept moist with 37°C sterile saline solution throughout the surgical intervention. The crush site was marked with a 10-0 nylon suture (Alcon). In the sham-operated group, the left sciatic nerve was treated in the same way except for the crush. Finally, the muscle and skin were sutured with 6-0 nylon and rats were allowed to recover spontaneously from anesthesia. After recovery, rats were housed in groups of three per cage and received buprenorphine (1mg/kg) for three days after surgery. To prevent autotomy, bitter nail polish was applied to each rat's left foot. During the study, animals were examined for signs of autotomy and contracture.

Fifty rats with sciatic nerve crush were randomly allocated into five groups (n=10). In the two experimental groups, the animals were treated daily with Memantine at the doses of 5 or 10 mg/kg within 7 days after surgery. These selected doses of Memantine had a neuroprotective effect in experimental autoimmune encephalomyelitis (28). Controls were injected with vehicle (DMSO) and the sham-operated group was subjected to the surgical procedure without the nerve crush.

The recovery of motor function was assessed by calculating the sciatic functional index (SFI) at 1, 3, 5, 7 and 9 weeks after crush injury.

SFI test was performed in a confined corridor (100×10×20 cm) with a dark box at the end. A white paper was placed on the floor of the corridor. Before the surgery, all rats were trained to walk in the corridor. Once the animals had learned to walk along the runway without stopping, their footprints were recorded. To record the footprints, the hind paws of rats were pressed down onto a finger paint-soaked sponge. Then animals were allowed to walk down the corridor leaving their hind footprints on the paper. The SFI value was calculated by putting the obtained data in the formula: $\text{SFI} = -38.3[(\text{EPL}-\text{NPL})/\text{NPL}] + 109.5[(\text{ETS}-\text{NTS})/\text{NTS}] + 13.3 [(\text{EIT}-\text{NIT})/\text{NIT}] - 8.8$, where EPL: the experimental paw length, NPL: the normal paw length, ETS: the experimental toe spread, NTS: the normal toe spread, EIT: the experimental intermediary toe spread and NIT: the normal intermediary toe spread (3). The SFI value varies from 0 to -100, with 0 corresponding to normal function and -100 indicating total impairment. When no footprints were measurable, the index score of -100 was given (10). In each walking track, three footprints were analyzed by a single observer and the average of the measurements was used in SFI calculations.

At 5 and 9 weeks postoperative, non-invasive compound muscle action potential (CMAP) recording was performed in all animals following anaesthesia by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). CMAPs were recorded in the gastrocnemius muscle by surface stimulation via the tendon-belly method (27), using an electrophysiological apparatus (CEPTU, England) with the PicoScope System Software. The sciatic nerve was stimulated by bipolar stimulating electrodes, which were placed on the skin premoistened with gel electrode, just between the

ischial tuberosity and major trochanter and parallel to the sciatic nerve. The active and reference monopolar needle electrodes were inserted into the mid-belly and muscle tendon surface, respectively. A ground electrode was clamped to the skin, between the stimulating and recording electrodes. Stimulations with durations of 0.02 ms were administered at gradually increasing intensity until a maximal CMAP response was obtained. The recording was repeated three times, and the amplitude and latency of CMAP were averaged for each rat. Normal CMAPs were measured from the contralateral uninjured sides. All acquired data were entered into the computer to calculate the electrophysiological parameters of the regenerated nerve.

At 9 weeks postoperative, following the electrophysiology study, the animals were deeply anaesthetized with an intraperitoneal injection of ketamine and xylazine cocktail, and the distal parts of the crushed site of the left sciatic nerves were harvested from each group. Nerve samples were fixed in 4% paraformaldehyde and post-fixed in 1% osmium tetroxide. After dehydration with ascending ethanol passages, the specimens were embedded and serial cross-sections of the distal zone for each nerve were cut. Cross sections of nerves were conducted starting 1 mm distally to the distal nylon suture (where the distal stump had been originally sutured) in order to allow visualization of regenerated fibers entering the distal nerve stump. For histomorphometric analysis, paraffin sections were stained with 1% toluidine blue (29). Axon counts, axon diameter and myelin thickness were calculated using the Image J program. The contralateral sciatic nerves were used as controls.

Muscle weight was measured to assess denervation atrophy at 9 weeks postoperative and after the electrophysiological test. The gastrocnemius muscles were harvested from both the experimental and contralateral (control) sides in each group. After bloodstain removal, the muscle was weighed while still wet using a digital scale (Sartorius, Germany). Then the gastrocnemius muscle mass ratio of the operated side to the contralateral side was calculated (muscle mass ratio = weight of experimental muscle/weight of contralateral muscle). The index percentage produced represented the recovery in denervation atrophy of the gastrocnemius muscle on the operated side, with approximately 100% gastrocnemius muscle index (GMI) indicating full recovery of the operated side (19).

Data were analysed with SPSS Statistics 16.0 software (SPSS Inc., Chicago, Illinois, USA). Statistical analysis was carried out using a one-way analysis of variance (ANOVA) to determine the significant differences between the five groups. Intergroup comparison of means was performed using a Tukey-Post hoc analysis. All data were expressed as mean \pm standard error of mean (SEM) and values of $P < 0.05$ were considered statistically significant.

RESULTS

Immediately after crushing the sciatic nerves (Figure 1), the compression areas were flattened but epineurium sheath continuity was preserved. All animals developed flaccid

paralysis of the operated foot and survived with no wound infection.

Functional Evaluation

According to statistical data, the mean SFI value in the sham-operated group was approximately (8.12 ± 0.79) throughout the study, indicating normal function. One week after sciatic nerve crush, the SFI values in all groups decreased dramatically to the lowest level (near to -100) with a significant difference compared to sham-operated animals, indicating complete loss of function ($P < 0.05$; Figure 2). Afterwards, all groups showed a time-dependent increase in SFI value with no significant differences between Memantine treatment and control groups in any week post-injury (Figure 2). Also, no significant difference was observed between Memantine treatment groups (5 and 10 mg/kg) at any week (Figure 2). At the 9th week, the SFI level of the Memantine treatment and

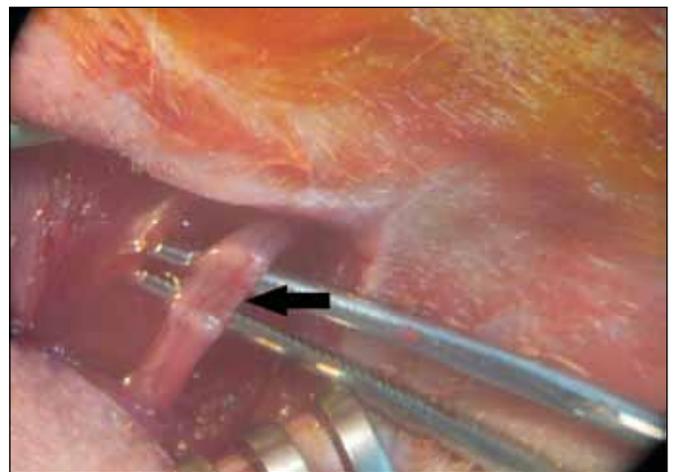


Figure 1: Picture showing the site of the sciatic nerve crush (arrow).

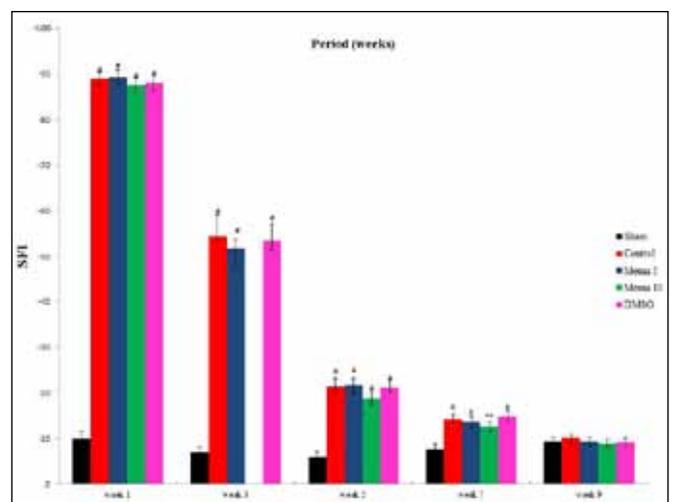


Figure 2: Sciatic function index (SFI) measured every other week after injury in each experimental group. Data were expressed as mean \pm SEM ($n = 10$). # $P < 0.001$ and * $P < 0.05$ vs. the sham group at the same time point.

control groups were not significantly different compared with the sham-operated group and SFI returned to the baseline values of the preoperative period (Figure 2). These results indicate that Memantine treatment did not affect recovery of sciatic nerve motor function.

Electrophysiological Evaluation

To assess the regeneration process, electrophysiological recordings were conducted at the 5 and 9 weeks endpoints. For this purpose, the CMAP peak amplitudes and CMAP onset latencies were measured. In all groups, the CMAP amplitudes increased and CMAP onset latencies decreased progressively with time (Figure 3). At 5 weeks post-injury, CMAP amplitude values showed a significant difference between Memantine treatment and control groups compared with the sham-operated control group ($P < 0.05$). However, no significant difference was observed between Memantine treatment and control groups (Figure 3). At the end of 9 weeks, there was no significant difference between the CMAP amplitude in any group (Figure 3). There was also no significant difference between the Memantine treatment and control groups in terms of CMAP onset latencies at the same time (Figure 4). However, CMAP onset latencies in the sham-operated group were significantly shorter than that recorded from the Memantine treatment and control groups ($p < 0.05$; Figure 4).

Histomorphometric Analysis

According to quantitative morphometric analyses of the regenerated nerves, regenerating myelinated axons in the distal to injury site were found to be densely populated, with thinner myelin in comparison with the sham-operated group, at 9 weeks after crush ($p < 0.05$; Table I). Increase in nerve fiber counts at the distal segment of the regenerated nerve may be due to axon-sprouting into the distal nerve (22). However, morphometric parameters such as the mean axonal

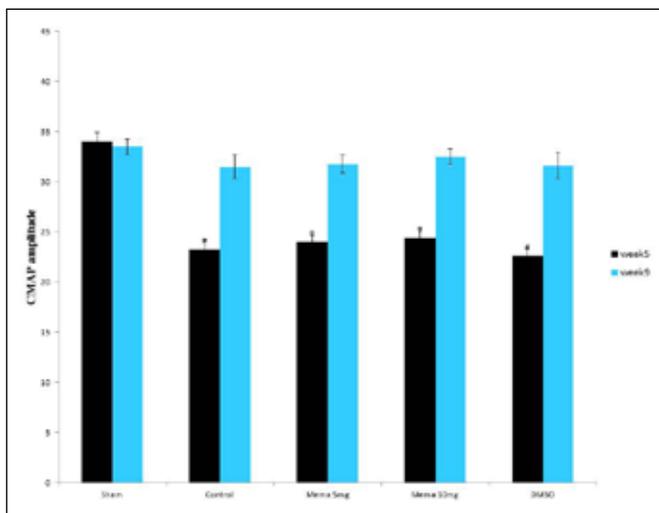


Figure 3: Representative results of CMAP amplitude measurements after proximal stimulation of operated and unoperated sciatic nerve at the 5th and 9th weeks post-injury. The data are shown as mean ± SEM (n = 10). # P < 0.001 and *P < 0.05 vs. the sham group.

number and myelin thickness showed no significant difference between the Memantine treatment and control groups (Table I).

Muscle Mass

At post-operative week 9, the mean ratios of gastrocnemius muscles weight were measured. The gastrocnemius muscles in the operated limbs exhibited atrophy compared with the contralateral side in all groups. The results showed that there was no significant difference in the severity of muscle atrophy between the Memantine treatment and control groups. Therefore, Memantine treatment could not reduce muscle atrophy (Figure 5).

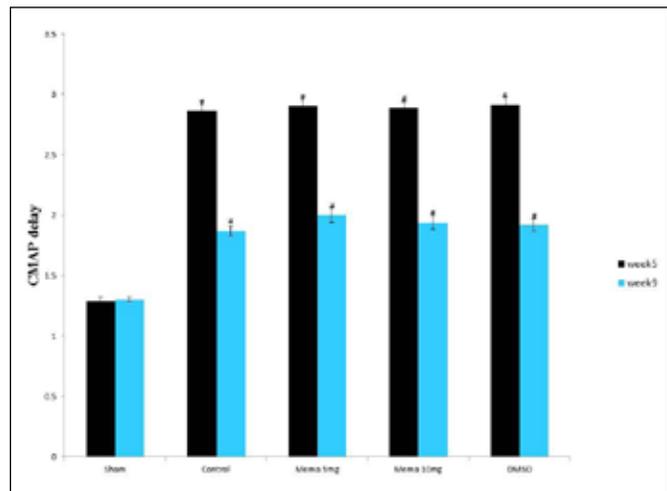


Figure 4: Representative results of CMAP delay measurements at the 5th and 9th weeks post-injury. The data are shown as mean ± SEM (n = 10). # P < 0.001 and *P < 0.05 vs. the sham group.

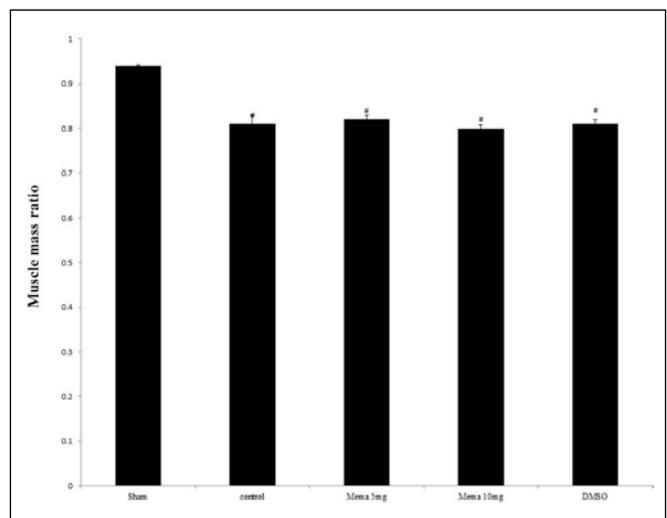


Figure 5: Gastrocnemius muscle mass ratio measurement. The gastrocnemius muscles of operated and unoperated sides were excised and weighed in the experimental groups at 9 weeks post-operatively. The data are shown as mean ± SEM (n = 10). # P < 0.001 and *P < 0.05 vs. the sham group.

Table I: Morphometric Analyses of Transverse Sections at the Sciatic Nerve Distal to Injury for Each of the Experimental Groups 9 Weeks Post-Injury

Groups	Number of fibers	Diameter of fibers	Diameter of axon	Thickness of myelin sheath
Sham	7594.75 ±92.64	6.29 ±0.08	3.73 ± 0.06	1.28 ± 0.02
Control	9567.12 ± 105.41	4.35 ± 0.07	2.84 ± 0.07	0.76 ± 0.01
Memantine 5 mg/kg	9605.75 ±90.61	4.27 ± 0.08	2.79 ± 0.05	0.74 ± 0.02
Memantine 10 mg/kg	9703.75 ±107.65	4.34 ± 0.11	2.86 ± 0.06	0.74 ± 0.03
DMSO	9584.25 ± 82.18	4.3 ± 0.08	2.8 ± 0.07	0.75 ± 0.02

Values are Shown as mean ± SD. # $P < 0.001$ and * $P < 0.05$ vs. sham group.

■ DISCUSSION

Peripheral nerve injuries make up 10% of all injuries and 30% of extremity injuries (33). Reinnervation time depends on the level of the lesion and whether the nerve division is far from the target organs. After intermediate-and low-level repairs, recovery was more useful than high-level repairs (33). The nature of the peripheral nerve demands a complex reparative process after nerve division. The goal of nerve repair is to bring the proximal and distal ends of the nerve, the fascicles or the fascicle groups together into close apposition without tension (8). Following nerve trauma, administration of neuroprotective agents is an appropriate strategy to control damage and promoting nerve regeneration process (38). Memantine is a non-competitive antagonist of the NMDA receptor with neuroprotective effect against some neurological disorders (16). In vitro studies using neuronal cell cultures showed that neuronal damage induced by glutamate was inhibited by Memantine (46). In vivo studies indicated that Memantine prevents the progression of neuronal loss in the amyotrophic lateral sclerosis and multiple sclerosis disease models (47). Furthermore, clinical trials showed the beneficial effects of Memantine for the treatment of AD (31) and PD (24). Some studies showed that Memantine might prevent the development of central sensitization and neuropathic pain (26, 37). Also, a neuroprotective effect of Memantine against rabbit model of optic nerve ischemia (18), rat model of retinal injury (48) and spinal cord injury (11) has been reported. The result of a recent study, conducted by Topdag et al. in 2015, suggested that Memantine improves functional recovery of the facial nerve after crush injury (40). On the other hands, our unpublished data showed that early administration of Riluzole, a sodium channel blocker and anti-glutamatergic agent, after sciatic nerve crush could delay motor function recovery despite its well documented neuroprotective effect in the central nervous system (36,44). We propose that the inhibitory effect of Riluzole on the sodium current has a dominant role in the sciatic nerve crush model rather than its antigitamatergic effect. Based on such findings, the present study was conducted to investigate the neuroprotective effect of Memantine on sciatic nerve crush injury in rat. Our results showed that daily parenteral administration of Memantine (5 and 10 mg/kg) immediately after sciatic nerve crush has no significant effect on nerve regeneration process rate and functional recovery quality. Memantine, a non-competitive antagonist

of the NMDA receptor, reduces pathological overactivity of NMDA receptors while allowing physiological activation (16,21). Peripheral nerve injury results in excess release of glutamate from primary sensory neurons into the dorsal horn of the spinal cord (15). Several recent studies have indicated that glutamate receptors may be involved in motoneuronal death following axotomy in neonatal rats. Motoneuronal death after axotomy in newborn rats can be augmented by NMDA application (23). Also, the dorsal horn content and basal release of the excitatory amino acids glutamate and aspartate are increased in rats that have experienced chronic constriction injury (CCI) to a sciatic nerve (35). Glutamate is the major transmitter of primary sensory afferents including nociceptors (4) and of excitatory interneurons in the dorsal horn of the spinal cord (39). Excessive glutamate release in dorsal horn of spinal cord results in overactivation of NMDA receptors and central sensitization (49). NMDA antagonists such as Memantine resulted in a decrease in mechanical hyperalgesia and mechanical allodynia in neuropathic rats (6,34). Since glutamate excitotoxicity is a major cause of neuronal death after ischemia and traumatic injury in the CNS (45), glutamate antagonists can reduce neural tissue damage via inhibiting glutamate excitotoxicity (30). However, according to our results, Memantine has no significant effect on regeneration process in the sciatic nerve crush model. The major neuroprotective effect of Memantine is due to its protective activity against NMDA receptor-mediated excitotoxicity (9). It seems that, glutamate excitotoxicity is less important in motor impairment due to sciatic nerve crush injury.

■ CONCLUSION

Our study showed that Memantine given after crush injury has no significant effect on motor function recovery in the sciatic nerve crush model in rat. However, neuroprotective effects of NMDA antagonists such as Memantine in traumatic injuries of CNS are well documented. These differences may be due to different neural degeneration process and recovery in CNS and PNS. It is clear that more research is needed to confirm these findings. However, peripheral nerve transection causes more severe damage and apoptosis due to glutamate excitotoxicity in neurons of the dorsal root ganglion and ventral horn of the spinal cord. We suggest studying the effect of Memantine on recovery from a sciatic nerve transection.

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