Morus alba Enhanced Functional Recovery After Sciatic Nerve Crush Injury

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Abstract: Problem statement: Traumatic nerve injury has been recognized as one of the problems commonly found in road traffic crashes. Therefore, searching for the effective substances for promoting the functional recovery of nerve after injury is in required. Accumulating lines of evidence show that free radicals generated after injury contribute the important role to retard functional recovery, thus the substance possessing anti-oxidant could facilitate functional recovery. Based on the effect of antioxidant to promote functional recovery of nerve after injury mentioned earlier, we hypothesized that Morus alba extract, a substance possessing anti-oxidant activity, should be able to facilitate the functional recovery of peripheral nerve after injury. Approach: To elucidate this issue, male Wistar rats, weighing 180-220 g, were orally given the aqueous extract of Morus alba at various doses ranging from 0.1, 1 and 10 mg kg\(^{-1}\) BW 5 days before and 21 days after sciatic nerve injury. Motor, sensory and sensorimotor coordination were observed every 3 days for 3 weeks by using De Medinacelli method, foot reflex withdrawal test and rotarod test, respectively. Results: The low dose of the extract significantly improved both sensory and motor functions after crush injury. Although sensory function recovers more sooner than the motor function, it fails to show full recovery within weeks. Thus, the present study demonstrates the potential of M. alba extract to enhance functional recovery after crush injury. Conclusion: In conclusion, Morus alba may serve as functional food to promote nerve recovery after injury. However, further studies about the possible active ingredient(s) and underlying mechanism(s) are required.

Key words: Morus alba, nerve crush injury, sciatic nerve, functional recovery

INTRODUCTION

At present, injuries from road traffic crashes have become a major public health and socio-economic problems. Traffic crashes usually induce traumatic nerve injury resulting in the disruption of intraneuronal circulation (Lundborg, 1988; Zochodne and Ho, 1990). This condition in turn induces demyelination, remyelination, axonal degeneration, axonal regeneration, focal, multifocal or diffuse nerve fiber loss and endoneural edema (Bagdatoglu et al., 2002; Zochodne and Ho, 1990). After injury, free radicals were elevated and produced more tissue damage and retarded the recovery process (Arslan et al., 2002; Bagdatoglu et al., 2002; Cinel et al., 2003; Talas et al., 2002).

Recently, accumulating lines of evidence showed that the substances exhibiting neuroprotective effect could improve nerve regeneration after nerve injury. Moreover, the ability to regenerate nerve was reported to be associated with the level of antioxidant such as vitamin E (Enrione et al., 1999). Numerous plant extracts possessing antioxidant activity such as proanthocyanidin-A2 were also reported to stimulate nerve regeneration and promote neurite outgrowth (Ambrogini et al., 1995). It has been proposed that these substances can increase the level of growth factor, a substance contributing the important role on nerve recovery after injury (Li and Ohizumi, 2004).

Morus alba Linn., or mulberry or Mohn is an economic plant in Northeast of Thailand. M. alba leaves were served as food for silk worm and indigenous vegetable for local people in this region. It was also used in traditional medicine for various medicinal purposes including therapeutic agents for asthma, bronchitis, cough, cold, constipation, epilepsy,

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headache, hyperglycemia, hypertension, vertigo, nervous stress, wound healing and anti-aging. In addition it was also used as a nourishing tonic medicine. Previous studies demonstrated that the M. alba leaves extract contained numerous substances possessing antioxidant (Andallu and Varadacharyulu, 2003). Based on the role of free radicals which retarded the functional recovery of nerve after injury and the benefit of substance possessing antioxidant to promote nerve recovery mentioned earlier, we hypothesized that Morus alba could facilitate the functional recovery after nerve injury. Hence, the present study was carried out to elucidate this issue.

MATERIALS AND METHODS

Plant material and extract: The crude extract of M. alba leaves was supported by Associate Professor Dr. Bungorn Sripanidkulchai, the Director of Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The descriptive processes were described as following. Mulberry leaves (M. alba strain, Burirum 60) were harvested, cleaned and brewed for 1 h in hot distilled water. The brewed solution was filtered and then the filtrate was lyophilized. The percent yield of extract was 16.54%. The dry extract was kept in airtight container at 4°C until used. The extract was suspended in distilled water just before used.

Preparation of the extract: The crude extract of M. alba leaves was dissolved in distilled water in order to obtain the desired concentration before the oral administration via intragastric tube once daily. Each animal in this study received the substance at the same volume.

Animal treatments: Young adult male Wistar rats, 8 weeks old, were used as experimental model. They were obtained from National Animal Center, Salaya. The weights of the animals on the first day of experiment were 180-220 g. They were randomly housed 5 per cage and maintained in 10:14 light: Dark cycle and given access to food and water ad libitum. All injections in this study were performed once daily between 8.00-9.00 am.

Surgical procedures: The animals were anesthetized. An incision was made at the thigh and the sciatic nerve was carefully exposed at a point immediately distal from the gluteus maximus muscle. The nerve was crushed for 30 sec using arterial forceps. A crush lesion was placed at the same site (mid-thigh) in both vehicle treated group and M. alba treated group.

Experiment protocols: The animals were randomly divided into 5 groups as described following:

Group 1: Sham operation group
Group 2: Vehicle plus nerve crush injury group: The rats in this group received distilled water orally once daily 5 days before and 21 days after the crush injury.
Group 3: M. alba 0.1 plus nerve crush injury group: The rats in this group received M. alba 0.1 mg kg⁻¹ BW orally once daily 5 days before and 21 days after the crush injury.
Group 4: M. alba 1 plus nerve crush injury group: The rats in this group received M. alba 1 mg kg⁻¹ BW orally once daily 5 days before and 21 days after the crush injury.
Group 5: M. alba 10 plus nerve crush injury group: The rats in this group received M. alba 10 mg kg⁻¹ BW orally once daily 5 days before and 21 days after the crush injury.

All animals were determined motor function using De Medina celli method, sensor motor coordination using rotarod and sensory function using foot reflex withdrawal every 3 days. In order to determine the possible mechanism of the extract, the optimum dose of M. alba was selected to study the effect of M. alba on the alteration of free radicals in sciatic nerve after crush injury.

Rotarod: In the rotarod test, an animal is placed on a rotating rod. The speed of rotation is gradually increased and the animal’s ability to remain on the rotating rod is recorded and recognized as endurance time. The purpose of the rotarod test is to assess the animal’s sensor motor co-ordination. The animals in each group were accessed rotarod test to determine the sensorimotor coordination every 3 days for 21 days after the crush injury of sciatic nerve.

Walking track test (De Medinacelli method): Walking track test was performed 3 days after surgery and every 3 days until the end of the experiment. The rats were first allowed in conditioning trials of 8.2×42 cm walking track. Paper was placed on the bottom of the track. The rat’s hind feet were dipped in ink. The rat was allowed to walk down the track and leaving its hind feet prints on the study. From the footprints, several measurements were undertaken and the so called Sciatic Function Index (SFI) was calculated by the equations in Fig. 1.
Fig. 1: Walking tract analysis (De Medinacelli method). The equations were used for calculating the toe spreading and foot print from the Ipsilateral paw (I) relative to the Contralateral (C) paw. Toe Spreading (TS) is a measure for the function of the intrinsic muscle of the paw. The print length (pl) reflects the function of soleus and gastrocnemius muscles, which are among others involved in raising of the ankle. The factors 100 1 and 196 set the TS and FP at -100% immediately after crush. The TS and FP are regarded as normal when they are within the range of -10 to 10%. Its: Inner toe spreading; ots: Outer toe spreading; pl: Print length

Foot reflex withdrawal test: The recovery of sensory function was measured by the foot reflex withdrawal test. The test was performed 3 days after surgery and every 3 days after the first measurement until the end of the experiment. The electric stimulation (0.1-10 volts) was carried out on the lateral side of the operated footsole. The animals which received sciatic nerve crush do not retract their paw upon skin contact with the electric stimulation. A healthy rat immediately withdraws its foot and spread its toes after stimulation. The lowest current which elicited this reflex at the operated side was recorded.

Determination of Malondialdehyde (MDA) level: Lipid peroxidation was measured by Assessing Malondialdehyde (MDA), an intermediary product of lipid peroxidation, using thiobarbituric acid and was expressed as nm MDA mg⁻¹ protein.

Determination of axonal density: After the end of the experiment, all animals were sacrificed and tissues were collected for histological study. The tissues were embedded in paraffin and cut at 10 μm thick. The sections were stained with hematoxylin and counter stained with eosin. The sections were analyzed using light microscopic and the axonal density was determined by using the program of Image Pro-plus 5.1.

Statistic analysis: All data were expressed as mean ± Standard Error of Mean (SEM) value and analyzed by One-Way ANOVA, followed by Post Hoc. The statistical difference was regarded when p-value<0.05.

RESULTS

The present results showed that sciatic nerve crush injury could alter foot print score as shown in Fig. 2. Our results have demonstrated that the animals which received M. alba leaves extract at dose of 0.1 mg kg⁻¹ BW show a significant improvement in motor function as shown in Fig. 3. The foot print score or sciatic nerve function index of the animals significantly increased after 15 days of post crush injury treatment (p<0.05; compared to vehicle plus crush injury). The prolonged treatment still continually improved foot print score until the end of experiment. In addition, the increasing doses of M. alba leaves extract to 1.0 and 10 mg kg⁻¹ BW failed to show significant changes. Moreover, Fig. 4 demonstrated that the toe spreading score also showed the same pattern of change as the foot print score. The animals which received the M. alba leaves extract at dose of 0.1 mg kg⁻¹ BW also showed the higher score than that of the vehicle treated group after 15 days of post crush injury treatment (p<0.05). The plant extract at this dose still showed continually improvement of this parameter until the end of the experiment (p<0.05; compared to vehicle plus crush injury). However, the Increasing dose further to 10 mg kg⁻¹ BW failed to show a significant improvement in this parameter.

The recovery of sensory function of the nerve after crush injury was assessed using foot reflex withdrawal test as the index. Figure 5 showed that after 12 days of post crush injury treatment, the animals which received M. alba leaves extract at dose of 0.1mg kg⁻¹ BW also showed a significant lower sensitivity to electrical stimuli compared to the vehicle treated group (p<0.05) and the significance was observed through the end of the experiment (p<0.05; compared to vehicle plus crush injury). Within the end of experiment, the significance was observed at all dosage range used in this study (p<0.05; compared to vehicle plus crush injury). Therefore, these findings suggested that the sensory function of sciatic nerve started to recover more sooner than the motor function.
Fig. 2: Foot prints of the animal after crush injury

Fig. 3: Effect of *M. alba* on the foot print score after sciatic nerve crush injury. Foot print scores of the animals were analyzed using the De Medinacelli method. The data were shown as mean ± SEM. (n = 6 per group). *: p<0.05; compared with vehicle group

Fig. 4: Effect of *M. alba* on the toe spreading score after sciatic nerve crush injury. The data were shown as mean ± SEM. (n = 6 per group). *: p<0.05; compared with vehicle group

In addition to the motor and sensory functions, the sensory-motor coordination started to show significant improvement after oral administration of *M. alba* at dose of 0.1 mg kg$^{-1}$ BW after 15 days of treatment post crush injury (p<0.05; compared to vehicle plus crush injury) and this significance was still observed until the end of experiment as shown in Fig. 6. The increasing dose of the plant extract to 1 mg kg$^{-1}$ BW showed the significant change of this parameter on the 21st day after the crush injury whereas no significant change was observed after the administration of the extract at dose of 10 mg kg$^{-1}$ BW through the whole duration of observation.

Fig. 5: Effect of *M. alba* on the recovery of sensory function of sciatic nerve. The electric stimuli at various intensities were applied to the sole of the injured foot to check the foot reflex withdrawal. The electrical intensity which elicited foot reflex withdrawal was recorded. The data were shown as mean ± SEM. (n = 6 per group). *: p<0.05; compared with vehicle group

Fig. 6: Effect of *M. alba* on the sensorimotor coordination in rotarod test. The data were shown as mean ± SEM. (n = 6 per group). *: p<0.05; compared with vehicle group

The present results also demonstrated that the low dose of the plant extract which significantly accelerated the functional recovery of both sensory and motor functions of sciatic nerve failed to show significant change in axonal density in sciatic nerve of both crush injury side and the contralateral side at 7 days after crush injury as shown in Table 1. However, the prolonged treatment of the *M. alba* leaves extract further to 14 and 21 days post crush injury significantly improved the axonal density at the crush injury side while no significance change was observed at the uninjured side (p<0.05; compared to vehicle plus crush injury).
Table 1: Effect of *M. alba* leaves extract on the alteration of axonal density at various duration after the sciatic nerve injury

| Treatment group | Injured side | Uninjured side |
|-----------------|--------------|----------------|
| **7 days after crush injury** | | |
| Sham | 338.50±2.97  | 340.67±3.16 |
| Vehicle | 245.50±1.41<sup>a</sup> | 347.33±7.16 |
| *M. alba* | | |
| 0.1 mg kg<sup>−1</sup> BW | 245.17±3.81<sup>a</sup> | 337.00±4.23 |
| **14 days after crush injury** | | |
| Sham | 338.00±3.64  | 341.33±2.22 |
| Vehicle | 296.00±4.14<sup>a</sup> | 336.50±2.25 |
| *M. alba* | | |
| 0.1 mg kg<sup>−1</sup> BW | 313.33±2.27<sup>a</sup> | 337.67±4.15 |
| **21 days after crush injury** | | |
| Sham | 337.33±2.98  | 339.33±2.95 |
| Vehicle | 305.33±7.57<sup>a</sup> | 339.67±3.89 |
| *M. alba* | | |
| 0.1 mg kg<sup>−1</sup> BW | 320.67±2.92<sup>a</sup> | 347.33±7.16 |

Rats were treated with either vehicle or *M. alba* at dose of 0.1 mg kg<sup>−1</sup> BW once daily via intragastric tube 5 days before and 7, 14 and 21 days after crush injury respectively. After the last treatment, the rats were sacrificed and isolated the sciatic nerve to determine axonal density. The cross sections of sciatic nerve (10 µm-thick) were stained with hematoxylin and eosin. Histological assessment was performed under light microscope at 40X magnification using Image Pro-plus 5.1 program. The data were presented as mean ± SEM. (n = 6 per group). : Compared with vehicle group at the injured side (p<0.05); *: Compared with sham operation at the injured side (p<0.05)

DISCUSSION

The present results have suggested that the sensory recovery function has occurred more sooner than the motor recovery functions of sciatic nerve. However, the motor function of the sciatic nerve shows full recovery within 21 days whereas the full recovery of sensory function is not observed within this period. The motor function recovery of sciatic nerve in this study was in agreement with the previous study which had reported that the motor function recovery in the foot showed fully recovery after sciatic nerve crush injury within 3 weeks (Vogelaar *et al.*, 2004). It has been reported that after nerve crush injury, the neuropathic pain which manifested as alldynia has been developed within the first 3 weeks (Attal *et al.*, 1994; Kingery *et al.*, 1994; Przewlocki *et al.*, 1999; Vogelaar *et al.*, 2004). Thus, the slow recovery process of the sensory nerve observed in this study may occur partly due to the development of neuropathic pain which takes much longer to resolve.

Previous studies had demonstrated that this condition was associated with sympathetic sprouting primarily around large neurons (Jones *et al.*, 1999; Shinder *et al.*, 1999; Zhong *et al.*, 1999; Hu and McLachlan, 2001). It has been found that the conditions mentioned earlier coincide with an up regulation of Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) in large mechanosensory neurons as opposed to small nociceptive neurons, which downregulate SP and CGRP (Woolf *et al.*, 1992; Hfktelt *et al.*, 1994; Noguchi *et al.*, 1994; Hu and McLachlan, 2001) induced by the Schwann cells and invading macrophage after injury.

The present results show that the oral administration of *M. alba* extract at dose of 0.1 mg kg<sup>−1</sup> BW can accelerate the functional recovery of both sensory and motor function of sciatic nerve. Based on the previous findings that Ginkgo biloba extract or EGb761 and *Centella asiatica* could improve the functional recovery of peripheral nerve by increasing the total number of myelinated axons in nerve (Hsu *et al.*, 2004; Soumyanath *et al.*, 2005), therefore, this study also determines the axonal density. Our results have also demonstrated the increase in density of axons in sciatic nerve after crush injury which in agreement with previous works.

Since extensive biochemical data have proposed the involvement of Oxygen Derived Free Radicals (ODFR) in the recovery process following neurotrauma as well as diabetic neuropathy, the present study is also undertaken to determine the level of l malondialdehyde, a lipid peroxidation process product, which used as indirect indicator for the alteration in the free radicals level in the nerve. The present results show that crush injury increases the malondialdehyde level in the nerve which indicates the elevation of free radicals. In addition, the present finding is corresponding with the previous study which reported that the level of antioxidant significantly decreased after crush injury and vitamine E supplement could accelerate the functional recovery after crush injury (Al Mouataery *et al.*, 1998).

Taken together, these data suggest that the acceleration of functional recovery induced by *M. alba* in this study may occur partly due to its antioxidant and its ability to increase the axonal density. The dose dependent response to *M. alba* extract was not observed in this study. This may occur because the plant extract used in this study is a crude extract consisting of various components; therefore, the increasing dose of the extract may probably increase the concentration of the components which exhibited their influences masking the effect of active component.

It has been previously reported that *M. alba* extract consisted of various components including flavonol glycosides particularly quercetin 3-(6-malonylglucoside) and rutin (Katsube *et al.*, 2006). These compounds were reported to reduce cellular stress in myotube culture (Young *et al.*, 2004).
therefore, the active component which accelerates the functional recovery of the sciatic nerve after crush injury in this study is probably related to these compounds. However, the identification of active compound is beyond the scope of this study and required further study.

CONCLUSION

In conclusion, we have clearly demonstrated that aqueous extract of *M. alba* leaves was capable of promoting the functional recovery of sciatic nerve after injury. Therefore, *M. alba* has the potential to be functional food for nerve injury repair application. However, further research is required to elucidate the possible active ingredient and the precise underlying mechanism.

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