Anti-edema effect of melatonin on spinal cord injury in rats

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Aim. To determine the anti-edema effects of melatonin on spinal cord injury (SCI) in rats.

Methods. A total of 150 adult male Sprague–Dawley rats were randomly allocated to the following three groups (n=50): a sham group which underwent laminectomy without dural compression; an SCI group, which underwent laminectomy followed by SCI and received saline i.p. immediately after injury and then daily for 2 days; an MT group, which underwent laminectomy followed by SCI and received a 100 mg/kg dose of melatonin i.p. immediately after SCI and then daily for 2 days. The cords were removed at 12, 24, 48 and 72 h after surgery in every group. Spinal cord edema was evaluated by determining the spinal cord water content. Expressions of AQP4 and GFAP positive cells in injured spinal cord were detected by immunohistochemical staining, and protein expressions of AQP4 and GFAP were detected by Western blotting.

Results. Spinal cord water content was obviously increased after SCI, which was maintained almost unchanged by melatonin treatment (100 mg/kg) at 12 h after injury but was significantly reduced from 24 h to 72 h. The expressions of AQP4 and GFAP increased in the injured spinal cord segments, which were decreased by melatonin treatment (100 mg/kg) between 24 h and 72 h after SCI.

Conclusions. Melatonin (100 mg/kg) had anti-edema effects after acute SCI probably by down-regulating the expression level of AQP4 protein, and it may eliminate astrocytic swelling after SCI through down-regulating the expression level of GFAP protein.

Key words: edema, melatonin, spinal cord injury, rats

Received: August 17, 2014; Accepted: March 10, 2015; Available online: April 24, 2015
<http://dx.doi.org/10.5507/bp.2015.012>

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INTRODUCTION

The annual incidence of spinal cord injury (SCI) is 11.5-53.4 cases/million population worldwide. It impairs motor, sensory and autonomic functions¹⁻³. The degree of dysfunction having direct effects on the the quality of life of the affiliated. For this reason, regenerating injured spinal cords is attractive in both clinical and basic medical research fields. As a complex cytological and biological process, repair of the spinal cord remains problematic. SCI is associated with spinal cord edema that readily leads to morbidity and mortality^{4,5} and adversely affects the postoperative outcome. For this reason, mitigating spinal cord edema⁶ would have a beneficial effect on the SCI (ref.⁷).

There are currently no specific drugs for SCI treatment. Despite many studies, neurological dysfunction that occurs at or below the level of the neurological insult can only be treated by the administration of methylprednisolone (MP) within 8 h after injury⁸⁻¹². However, MP has side-effects that make its use in clinical practice

controversial. On the other hand, Melatonin (N-acetyl-5-methoxytryptamine), which is the main secretory product of the pineal gland that functions as a biological synchronizer in the circadian rhythm, has high antioxidant activities¹³⁻¹⁸.

Aquaporins play important roles in water transport in many types of cells. Aquaporin-4 (AQP4) protein is highly expressed in the central nervous system (CNS) (ref.^{19,20}), the expression changes of which are associated with spinal cord edema and the permeability of blood-spinal cord barrier^{21,22}. The expression of AQP4 in injured spinal cords changes in a similar manner to the water content of spinal cord^{23,24}. The swelling of astrocytes for which GFAP is a specific marker also plays a crucial role in cellular edema after acute SCI.

The effect of melatonin on spinal cord edema after SCI remains unknown to date. The aim of this study was to investigate the anti-edema effects of melatonin (100 mg/kg) on spinal cord edema after acute SCI and its underlying mechanism.

MATERIALS AND METHODS

Animals and surgical technique

After acceptance of the local ethics committee, the study was started at the Severe Wound and Trauma Laboratory of General Hospital of Shenyang Military Area Command of Chinese PLA. Efforts were made to minimize animal suffering and to reduce the number of animals used in experimental groups. Adult male Sprague-Dawley (SD) rats (205-225 g) were used for the study protocol. The animals were housed in cages (4 per cage) before surgery. Water and food were continuously available during preoperative and postoperative periods. Twelve-hour daylight and darkness cycles were used before and after surgery. Room temperature was kept constant (26 ± 2) °C.

Rats were anesthetized with chloral hydrate (300 mg/kg body weight). Breathing was continued spontaneously with room air. The animals were supine on the operating table. Under sterile conditions, a midline dorsal incision was made and laminectomy was performed at the T12-level vertebra, leaving the dura intact. Strict bleeding control was held by using bone wax and bipolar coagulator. An aneurysm clip was applied extradurally for 5 min on thoracic spinal cord at room temperature. After careful removal of the clip, paravertebral fascia and skin were sutured separately with silk stitches. A complete closure of surgical wound was achieved. Complicated cases, such as dural tearing or inadvertent spinal injuries, were excluded from the study. Rats were examined in the early postoperative period and settled in cages separately. Food and water were provided. The urinary bladders were pressed three times a day.

Experimental groups

A total of 150 rats were randomly allocated to the following three groups (n=50): Sham group, which underwent laminectomy alone, without dural compression; SCI group, which underwent laminectomy followed by SCI and received saline i.p. immediately after injury and then daily for 2 days; MT group, which underwent laminectomy followed by SCI and received a 100 mg/kg dose of melatonin (Hangzhou Gosun Biotechnologies Co., Ltd., China) i.p. immediately after SCI and then daily for 2 days. Melatonin was dissolved in ethanol and further diluted in physiological saline. The final ethanol concentration was 5%. At 12, 24, 48 and 72 h following trauma, five rats from each group were used for analysis of the spinal cord water content, and another five for Western blot analysis, respectively. At 24 and 72 h after operation, five rats from each group were used for immunohistochemical (IHC) staining.

Determination of spinal cord water content

Spinal cord edema was evaluated by determining the spinal cord water content. The cords were removed at 12, 24, 48 and 72 h after surgery in each group. The spinal cords were 1 cm long segments centered at the injury epicenter. After determination of the wet weight, the injured

spinal cords were dried for 48 h at 80 °C to determine the dry weight. Water content in spinal cord tissue was calculated as (wet weight - dry weight)/ wet weight $\times$ 100%.

IHC staining

IHC staining was performed, to investigate the expressions of the AQP4 and GFAP positive cells in the injured spinal cord at 24 and 72 h after injury. To remove the cords, animals were reanesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffered saline (0.1 M PBS, pH 7.4). Then the cords were further exposed from vertebrae T10 to L1 so that 1 cm long segments centered at the injury site were removed and fixed in the same fixative for another 24 h at 4 °C. The fixed cords were dehydrated and embedded in paraffin sections. Axial tissue sections (10 μ m) were cut with a microtome at 3 mm upper of the SCI epicenter. Sections were deparaffinized and treated with 3% H₂O₂ for 15 min to block endogenous peroxidase. The sections were exposed to normal goat serum for 30 min, and then incubated with the following primary antibodies overnight at 4 °C: rabbit polyclonal anti-AQP4 antibody (diluted 1:150, Santa Cruz Biotechnology, Inc.) or mouse monoclonal anti-GFAP antibody (diluted 1:400, Sigma). After washing in PBS, the sections were incubated with the appropriate secondary antibody (ZSGB-BIO, China) at 37 °C for 15 min. Immunolabelling was visualized as brown, using diaminobenzidine, with hematoxylin counterstain. The number of AQP4 or GFAP positive cells in the gray matter of the sections was counted in a rectangular area of 190 $\times$ 340 μ m² by two observers (unaware of the experimental groups). The results counted by two observers were averaged to obtain the final count for the section.

Western blot

Western blot analysis was carried out to investigate the protein expressions of AQP4 and GFAP in the injured spinal cords at 12, 24, 48 and 72 h after injury. Spinal cord samples were 1 cm long and were homogenized in RIPA buffer. After homogenization, the samples were centrifuged at 17,000 g for 15 min at 4 °C. The protein concentration of soluble materials was determined by the Coomassie G250 Binding method. The protein lysates were fractioned on 12% SDS-polyacrylamide gels, followed by transfer to PVDF membranes. The membranes were blocked with 5% skimmed milk for 2 h and then incubated with primary polyclonal antibody anti-AQP4 (dilution 1:400, Santa Cruz Biotechnology, Inc.), GFAP (dilution 1:200, Sigma) or β -actin (dilution 1:2000, Santa Cruz Biotechnology, Inc.) overnight at 4 °C, followed by corresponding second antibody for 2 h at room temperature. The EC3 Imaging System (UVP Inc. Upland, CA, USA) was used to catch up the AQP4, GFAP protein bands and β -actin bands, and the optical density of every band was measured using an Image J software (NIH, Bethesda, MD, USA). To determine the expression levels of AQP4 and GFAP, the value of the protein was normalized to the corresponding β -actin band.

Statistical analysis

SPSS 16.0 software was used for the statistical analysis. Data presented as Means $\pm$ SD. One-way analysis of variance was performed to determine the differences among the groups for spinal cord water content, AQP4 and GFAP level. A p-value less than 0.05 was considered statistically significant.

RESULTS

Effects of melatonin on spinal cord water content

The spinal cord water content is shown in Fig. 1. The spinal cord water content after injury was significantly increased in the SCI group compared with that of Sham group at 12, 24, 48 and 72 h after injury. The spinal cord water content after injury was significantly reduced in the melatonin group compared with that of SCI group at 24, 48 and 72 h after injury, whereas it did not change significantly at 12 h after injury.

Effect of melatonin on AQP4 expression

The expression of AQP4 was detected using IHC and Western blot methods. The IHC results of AQP4 are shown in Fig. 2. AQP4 positive cells were strongly expressed in gray matter, around capillaries and in radial astrocytes. The number of AQP4 positive cells in the SCI group significantly exceeded that of the Sham group at 24 and 72 h after injury ($P < 0.05$). In the MT group, the number of AQP4 positive cells was significantly down-regulated at 24 and 72 h after injury compared with that of SCI group ($P < 0.05$).

The representative Western blot gels for AQP4 (34 kDa) and β -actin (42 kDa) are shown in Fig. 3. The

Fig. 1. Spinal cord water contents at 12, 24, 48 and 72 h after injury. The figure shows a significant increase in the spinal cord water content in SCI group compared with that of Sham group at 12, 24, 48 and 72 h after injury, while a significant reduction was observed in MT group compared with that of SCI group at 24, 48 and 72 h after injury. Data present mean $\pm$ SD ($n=5$, * $P < 0.05$ between Sham group and SCI group, # $P < 0.05$ between MT group and SCI group).

level of AQP4 was quantified and normalized to β -actin. AQP4 was expressed at low level in the Sham group and significantly increased at 12, 24, 48 and 72 hs after injury ($P < 0.05$, Fig. 3A–D). The level of AQP4 in the melatonin group plummeted compared with that of SCI group at 24, 48 and 72 hs after injury ($P < 0.05$, Fig. 3B–D) but 12 hs after injury, there were no significant differences between the SCI and MT groups ($P > 0.05$, Fig. 3A).

Effect of melatonin on GFAP expression

The IHC results of GFAP are shown in Fig. 4. The number of GFAP positive cells was significantly increased in the SCI group compared with that of Sham group at 24 and 72 hrs after injury ($P < 0.05$). In the MT group, the number of GFAP positive cells was obviously decreased at 24 and 72 hrs after injury compared with that of SCI group ($P < 0.05$).

With Western blot analysis, the IDVs of GFAP compared with β -actin are shown in Fig. 3. GFAP level was low in the Sham group and significantly increased at 12, 24, 48 and 72 h after injury ($P < 0.05$, Fig. 3A–D). Compared with SCI group, the expression level of GFAP protein in MT group obviously dropped at 24, 48 and 72 h after injury ($P < 0.05$, Fig. 3B–D). There were no

Fig. 2. Changes of AQP4 positive cells expression by IHC method at 24 and 72 h after injury (A: 24 h of Sham group, B: 24 h of SCI group, C: 24 h of MT group, D: 72 h of Sham group, E: 72 h of SCI group, and F: 72 h of MT group). The number of AQP4 positive cells in gray matter regions in SCI group was significantly up-regulated compared with that in Sham group at 24 and 72 h after injury. The number of AQP4 positive cells in MT group was significantly down-regulated compared with that in SCI group at 24 and 72 h after injury. Data present mean $\pm$ SD ($n=5$, * $P < 0.05$ between Sham group and SCI group, # $P < 0.05$ between MT group and SCI group, Scale bar=50 μ m).

Fig. 3. Western blot analysis for AQP4 and GFAP protein levels at 12 (A), 24 (B), 48 (C) and 72 h (D) after injury. AQP4 was detected at 34 kDa, GFAP was detected at 50 kDa and the loading control β -actin at 43 kDa. The graph showed significantly up-regulation of AQP4 and GFAP expression level in SCI group at 12, 24, 48 and 72 h after SCI. Treatment of melatonin (100 mg/kg) could markedly decrease AQP4 and GFAP levels in the spinal cord at 24, 48 and 72 h after injury. There were no significant differences in AQP4 and GFAP protein levels between SCI group and melatonin group at 12 h post-injury. Bars represent mean $\pm$ SD (n=5, * P <0.05 between Sham group and SCI group, # P <0.05 between SCI group and melatonin group).

Fig. 4. Changes of GFAP positive cells expression using IHC method at 24 and 72 h after injury (A: 24 h of Sham group, B: 24 h of SCI group, C: 24 h of MT group, D: 72 h of Sham group, E: 72 h of SCI group, and F: 72 h of MT group). The number of GFAP positive cells in gray matter regions in SCI group was significantly up-regulated compared with that of Sham group at 24 and 72 h after injury. The number of GFAP positive cells in MT group was significantly down-regulated compared with that of SCI group at 24 and 72 h after injury. Data present mean $\pm$ SD ($n=5$, * $P<0.05$ between Sham group and SCI group, # $P<0.05$ between melatonin group and SCI group, scale bar=50 μ m).

significant differences between the SCI group and MT group at 12 hs after injury ($P>0.05$, Fig. 3A).

DISCUSSION

In previous studies, SCI has been effectively treated with 50 or 100 mg/kg melatonin²⁵⁻²⁷. Gül et al. reported the neuroprotective effects of melatonin on experimental SCI in rats²⁸. The 100 mg/kg group had better outcomes than the 50 mg/kg group did. Therefore, we chose 100 mg/kg melatonin to treat SCI.

Two foremost studies have shown the highly beneficial effects of melatonin on experimental SCI (ref.^{29,30}). Both studies used similar weight drop injury models, with the first 50 g/cm and the other 25 g per 10 min. However,

dose regimens were significantly different in these studies: 100 and 2.5 mg/kg. The first study concluded that melatonin had significant protective effects mainly on myelin sheaths but also on nucleus and mitochondria. Similarly, the other study suggested that melatonin could protect against oxidative damage and reduce neutrophil-induced toxicity.

This study demonstrated that melatonin (100 mg/kg) treatment exerted good therapeutic effects following SCI, managing to decrease the spinal cord water content, AQP4 level and GFAP level.

SCI can result in serious disability, sensory disorders, paralysis, other neurologic deficits, and death³¹. To this end, it is necessary to understand the pathophysiology of the acute phase following SCI and to develop effective therapeutic interventions. Commonly, SCI is initiated by a

primary injury that causes mechanical compression of the spinal cord, followed by a secondary injury that induces considerable apoptotic cell death³². Secondary damage after SCI includes edema, altered blood flow, and changes in microvascular permeability^{33,34}. Traumatic injury to the spinal cord is often associated with edema that is predominant in the gray matter^{19,35}. This progressive, active spread of damage begins within minutes after initial injury and continues for weeks³⁶. In this study, we used the rat model of SCI to examine the protective effects of melatonin (100 mg/kg) on spinal cord edema from 12 to 72 h after SCI.

The spinal cord water content was obviously increased after SCI, which was maintained almost unchanged by melatonin treatment (100 mg/kg) at 12 h after injury but was significantly reduced from 24 hrs to 72 hrs. Therefore, melatonin effectively reduced SCI-induced spinal cord edema after a latency period and for the duration of the observation. As we described before, the pathophysiology of SCI comprises primary and secondary damage. Primary damage includes microvascular bleeding that also increases the spinal cord water content. The increase of spinal cord water content at 12 h after SCI may be ascribed to both primary and secondary damage, and melatonin treatment was unable to mitigate primary damage. The therapeutic effects of melatonin are mainly on the secondary damage.

We used IHC and Western blot methods to describe the expression levels of AQP4 and GFAP proteins after SCI. AQP4, as a member of the AQP family, is widely expressed in the nervous system. Previous studies showed the colocalization of AQP4 with GFAP in double labeling by using confocal microscopy^{37,38}. Badaut et al. reported that AQP4 was found in astrocyte foot processes around capillaries in the human brain³⁹. Oshio et al. found that AQP4 was intensely stained throughout the gray matter in spinal cord, especially in the capillary-surrounding astrocytic end-feet¹⁹. In particular, AQP4 and spinal cord edema are positively correlated^{40,41}. AQP4 in spinal cord is expressed in glial cells throughout the gray matter and glial foot processes adjacent to the spinal capillary endothelium¹⁹. In this study, AQP4 expression was significantly increased from 24 to 72 h after SCI, which was obviously decreased by melatonin treatment (100 mg/kg). Accordingly, melatonin (100 mg/kg) may exert anti-edema effects from 24 to 72 h after SCI by decreasing the AQP4 level. Conceivably, down-regulating AQP4 expression by melatonin (100 mg/kg) treatment may be conducive to alleviating the spinal cord edema in SCI rats.

Liang et al. demonstrated that cellular edema was important after CNS injury. Astrocytes are much more prone to swelling than neurons⁴², for which GFAP is a specific marker⁴³. In this study, melatonin (100 mg/kg) could obviously decrease the GFAP protein level in the spinal cord from 24 to 72 h after SCI. This may play a role in eliminating astrocytic swelling after SCI. In conclusion, the expressions of AQP4 and GFAP increased in the injured spinal cord segments. Melatonin treatment (100 mg/kg) decreased AQP4 and GFAP levels in spinal cord segments between 24 h and 72 h after SCI, implying that melatonin (100 mg/kg) had anti-edema effects after acute

SCI probably by down-regulating the expression level of AQP4 protein. Furthermore, melatonin (100 mg/kg) may eliminate astrocytic swelling after SCI through down-regulating the expression level of GFAP protein. More studies are needed to analyze the possible therapeutic effects of melatonin on SCI patients.

ACKNOWLEDGEMENT

Author contributions: XL: manuscript writing; YW: data collection and data analysis; JY: data interpretation; YL: statistical analysis; DZ: literature search; MH, LX: study design.

Conflict of interest statement: None declared.

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