Melatonin lowers edema after spinal cord injury

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Abstract
Melatonin has been shown to diminish edema in rats. Melatonin can be used to treat spinal cord injury. This study presumed that melatonin could relieve spinal cord edema and examined how it might act. Our experiments found that melatonin (100 mg/kg, i.p.) could reduce the water content of the spinal cord, and suppress the expression of aquaporin-4 and glial fibrillary acidic protein after spinal cord injury. This suggests that the mechanism by which melatonin alleviates the damage to the spinal cord by edema might be related to the expression of aquaporin-4 and glial fibrillary acidic protein.

Key Words: nerve regeneration; spinal cord injury; melatonin; edema; aquaporin; glial fibrillary acidic protein; immunohistochemistry; western blot assay; neural regeneration

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Introduction
Every year between 11.5 and 53.4 million people in the world are reported to suffer from spinal cord injury (SCI), which harms the autonomic, motor and sensory functions (Nakanishi et al., 2014; Wang et al., 2014). A patient’s quality of life is strongly associated with the degree of the injury to the spine. Currently, there is no good way to treat SCI, so it is important to investigate possible targets in the field of basic medicine for SCI (Wang et al., 2007; Zhang et al., 2014). However, the complexity of the biological and cytological processes of repairing the spinal cord poses many challenges.

SCI is correlated with spinal cord edema, which if unchecked can lead to severe damage and death (Leypold et al., 2008; Fujiki et al., 2014). Previous studies have proved that the outcomes after SCI are affected greatly by the progress of spinal cord edema, and an ideal strategy includes a reduction of spinal cord edema in vivo (Verkman et al., 2011; Yang et al., 2011). Spinal cord edema is often long lasting and therapy resistant and thus poses a main challenge for neurosurgeons (Pierrefiche et al., 1993; Tan et al., 1993; Reiter et al., 1995; Wang et al., 2009).

So far, there is no particular medicine for treating SCI. Although there has been much research, until now the only clinical pharmacological treatment to improve the neurological dysfunction following SCI was methylprednisolone, which had to be administered within 8 hours of SCI (Bracken et al., 1990, 1992, 1997, 1998). However, the side effects of methylprednisolone make its use to treat SCI controversial.

In many cell types, aquaporins have a significant function in transporting water (Oshio et al., 2004; Saadoun et al., 2010). Previous research has confirmed that the changes in aquaporin-4 are strongly linked with the blood-spinal cord barrier and its permeability in spinal edema. However, if aquaporin-4 expression changes in the injured spinal cord, this will cause changes in water content in the spinal cord (Xu et al., 2008; Wang et al., 2009). Astrocyte swelling plays an important role in cellular edema after acute SCI. Glial fibrillary acidic protein (GFAP) is a specific marker of astrocytes.

Melatonin (N-acetyl-5-methoxytryptamine), a major secretory product from the pineal gland, not only acts as a synchronizer for the circadian rhythm but also has powerful antioxidant activities. The effect of melatonin on spinal cord edema is poorly understood. This study sought to survey the underlying function of melatonin (100 mg/kg) on spinal edema in a rat model of SCI and the mechanisms of that melatonin action.

Materials and Methods

Animals
The Severe Wound and Trauma Laboratory of General Hospital of Shenyang Military Area Command of Chinese PLA, Shenyang, China (license No. SYXK (Liao) 2006-0002) provided 150 clean, healthy, female, Sprague-Dawley rats aged 10–11 weeks and weighing 250 ± 22 g. Animal experiments were approved by the Ethics Committee of General Hospital of Shenyang Military Area Command of Chinese PLA in China. Every effort was made to reduce pain and as few rats as possible were used during experiments. The rats were kept four to a cage. Rats were given water and food libitum under conditions of 12-hour light/dark cycles at 26 ± 2°C.

Model establishment
Rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and they breathed air naturally and evenly. The rats...
were prone on the operating table. A medial dorsal incision was made. A laminectomy was conducted. T13 vertebra and the dura were intact. Bone wax and a bipolar coagulator were used to stop the bleeding. The thoracic spinal cord was clipped with an aneurysm clip for 5 minutes at room temperature. After removing the clip carefully, silk stitches were used to sew the paravertebral fascia and the skin respectively. Those rats that had complications were not included in the experiment. The healthy adult rats were carefully observed after surgery, and placed in separate cages. The rats were supplied with food and water. Their urinary bladders were pressed three times every day.

Animal groups
The 150 rats were equally and randomly divided into three groups. The sham surgery group only received laminectomy without dorsal compression. The SCI group received laminectomy after SCI and the rats were injected with normal saline i.p. instantly after injury for 2 days. Melatonin group received laminectomy after SCI and the rats were administered 100 mg/kg melatonin (Solarbio Biotechnology, Beijing, China) i.p. promptly after SCI for 2 days (Wu et al., 2014). The melatonin was dissolved in anhydrous ethanol, and then diluted with normal saline to a concentration of 12.5 mg/mL. Five rats from each group were used to analyze the water content in the spinal cord. An additional five rats from each group were used for western blot assays, which were performed separately at 12, 24, 48, and 72 hours post SCI (Ma et al., 2010). After the operation, five rats from each group were selected for immunohistochemical staining at 24 and 72 hours.

Measurement of water content in the spinal cord
Spinal edema was evaluated by determining the water content in the spinal cord. At 12, 24, 48, and 72 hours after surgery, spinal cords were removed from five rats in each group. One-cm lengths of crushed spinal cord were weighed for their wet weights. Then the injured spinal cord was dried at 80°C for 48 hours so that water content could be calculated by wet weight – dry weight/wet weight × 100%.

Immunohistochemical staining
Immunohistochemical staining was used to explore the levels of aquaporin-4 and GFAP in the spinal cord 24 and 72 hours after SCI. Rats were anesthetized, perfused transcardially with saline, and fixed with 4% paraformaldehyde. The spinal cord was exposed from vertebra T10 to L1. A 1-cm long segment was obtained and placed at 4°C for 24 hours, dehydrated and immersed in paraffin. Tissue at 3-µm thickness were sliced into 10-µm axial sections with a microtome. These sections were treated with 3% H2O2 for 15 minutes to block endogenous peroxidase, incubated with primary antibody rabbit anti-aquaporin-4 polyclonal antibody (1:150; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) or mouse anti-GFAP monoclonal antibody (1:400; Beijing Biosynthesis Biotechnology Co., Ltd.) at 4°C overnight. The sections were washed with PBS, and then incubated with secondary antibody goat anti-rabbit IgG (1:400; Beijing Biosynthesis Biotechnology Co., Ltd.) for 15 minutes at 37°C. Sections were visualized using diaminobenzidine, and observed under a BX43 microscope (x 200; Olympus, Tokyo, Japan). The number of positive cells in a 190 µm × 340 µm rectangular area of the gray matter was counted by two observers independent of the experimenters.

Western blot assay
At 12, 24, 48 and 72 hours after injury, changes in aquaporin-4 and GFAP were observed by western blot assay. One-cm lengths of spinal cord were homogenized in radio-immune precipitation assay buffer, and then centrifuged at 17,000 x g for 15 minutes at 4°C. The Coomassie G250 binding method was used to determine the protein concentration of the soluble material. The protein lysates were transferred to polyvinylidene fluoride membranes (Beijing Biosynthesis Biotechnology Co., Ltd.). The membranes were blocked with 5% skim milk for 2 hours, and then incubated with polyclonal antibody rabbit anti-rat aquaporin-4 (1:400; Solarbio Biotechnology, Beijing, China), rabbit anti-rat GFAP (1:200; Solarbio Biotechnology) or β-actin (1:2,000; Solarbio Biotechnology). Samples were then incubated with goat anti-rabbit IgG (1:2,000; Beijing Biosynthesis Biotechnology Co., Ltd.) for 2 hours at room temperature. Aquaporin-4, GFAP and β-actin antibody bands were tracked by the EC3, an image-forming system (UVP Inc., Upland, CA, USA). The optical density was measured by ImageJ software (NIH, Bethesda, MD, USA). The expression levels of aquaporin-4 and GFAP in each group were determined. The value of the protein was normalized as its ratio to the sham.

Statistical analysis
The data were expressed by the mean ± SD, and analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance and the least significant difference test were used to determine the difference in water content. P < 0.05 was considered statistically significant.

Results
Melatonin effects on water content in rats with spinal cord injury
Compared with the sham surgery group, the water content was significantly higher in the SCI group (P < 0.05). Compared with the SCI group, the water content was significantly lower in the melatonin group at 24, 48 and 72 hours after surgery (P < 0.05). However, the water content in the spinal cord did not change significantly at 12 hours after injury (Figure 1).

Melatonin effects on aquaporin-4 expression
Immunohistochemical staining showed strong immunoreactivity in the gray matter. As shown in Figure 2, the number of aquaporin-4-immunoreactive cells was significantly higher compared with the sham surgery group at 24 and 72 hours after surgery (P < 0.05). However, there were fewer aquaporin-4-immunoreactive cells in the melatonin group than in the SCI group (P < 0.05). Figure 3 shows representa-
tive western blots for aquaporin-4 (34 kDa) and β-actin (42 kDa). The levels of aquaporin-4 to β-actin were quantified and normalized.

A western blot assay showed a low level of aquaporin-4 expression in the sham surgery group. Aquaporin-4 expression significantly increased at 12, 24, 48 and 72 hours after injury in the model group ($P < 0.05$; Figure 3A–D). Compared with the SCI group, aquaporin-4 expression was significantly lower in the melatonin group at 24, 48 and 72 hours after injury ($P < 0.05$, Figure 3B–D). No significant difference in aquaporin-4 expression was detected between the SCI group and melatonin group at 12 hours ($P > 0.05$; Figure 3A).

**Effects of melatonin on GFAP expression**

GFAP-positive cells in the SCI group were more numerous than in the sham surgery group at 24 and 72 hours after surgery. Conversely, the quantity of GFAP-immunoreactive cells was significantly lower in the melatonin group compared with the SCI group ($P < 0.05$; Figure 4).

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**Tables and Figures**

**Figure 1**: Melatonin effects on water content in rats with spinal cord injury.
- *P* < 0.05, vs. sham surgery group. #P < 0.05, vs. SCI group. Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance and the least significant difference test). SCI: Spinal cord injury.

**Figure 2**: Changes of aquaporin-4-immunoreactive cells at 24 and 72 hours after injury.
- (A) Immunohistochemical staining, optical microscope, × 200. (A) Arrows: Aquaporin-4-immunoreactive cells. (B) Changes in the number of aquaporin-4-immunoreactive cells. *P < 0.05, vs. sham surgery group; #P < 0.05, vs. SCI group. Data are expressed as the mean ± SD ($n = 5$, one-way analysis of variance and the least significant difference test). SCI: Spinal cord injury.

**Figure 3A–D**: Changes in the number of aquaporin-4-immunoreactive cells over time. (A) Immunohistochemical staining (optical microscope, × 200). Arrows: Aquaporin-4-immunoreactive cells. (B) Number of aquaporin-4-immunoreactive cells. *P < 0.05, vs. sham surgery group. #P < 0.05, vs. SCI group. Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance and the least significant difference test). SCI: Spinal cord injury.

**Figure 4**: Changes in the expression of GFAP-immunoreactive cells at 24 and 72 hours after injury.
- (A) Immunohistochemical staining (optical microscope, × 200). Arrows: GFAP-immunoreactive cells. (B) Changes in the number of GFAP-immunoreactive cells. *P < 0.05, vs. sham surgery group. #P < 0.05, vs. SCI group. Data are expressed as the mean ± SD ($n = 5$; one-way analysis of variance and the least significant difference test). SCI: Spinal cord injury; GFAP: glial fibrillary acidic protein.
Figure 3 Western blot assay for AQP4 and GFAP protein levels at 12 (A), 24 (B), 48 (C) and 72 hours (D) after injury.
The graph showed significant up-regulation of AQP4 and GFAP expression in the SCI group at 12, 24, 48 and 72 hours after SCI. Treatment of melatonin (100 mg/kg) could markedly decrease AQP4 and GFAP levels in the spinal cord at 24, 48 and 72 hours after injury. *P < 0.05, vs. sham surgery (sham) group; #P < 0.05, vs. SCI group. Data are expressed as the mean ± SD (n = 5, one-way analysis of variance and the least significant difference test). SCI: Spinal cord injury; AQP4: aquaporin-4; GFAP: glial fibrillary acidic protein.
A western blot assay showed that aquaporin-4 expression was low in the sham surgery group, but increased significantly at 12, 24, 48 and 72 hours after injury \((P < 0.05; \text{Figure 3A–D})\). Compared with the SCI group, the level of GFAP was significantly lower in the melatonin group at 24, 48 and 72 hours after injury \((P < 0.05; \text{Figure 3B–D})\). However, there was no significant difference between the SCI and melatonin groups for values of GFAP or aquaporin-4 at 12 hours after injury \((P > 0.05; \text{Figure 3A})\).

**Discussion**

Many previous studies have confirmed the effect of melatonin \((50 \text{ or } 100 \text{ mg/kg})\) on treating SCI \((\text{Liu et al., 2004; Esposito et al., 2009; Lee et al., 2011})\). Gül et al. \((2005)\) studied the effects of different doses of melatonin in rats with SCI. Their study used melatonin at 50 and 100 mg/kg to treat SCI in rats, and found that the 100 mg/kg dose was more effective than the 50 mg/kg dose.

There are two studies that show highly beneficial effects of melatonin on SCI \((\text{Fujimoto et al., 2000; Kaptanoglu et al., 2000})\). Both studies used similar weight drop injury models, but Fujimoto et al. used 50 g-cm, and Kaptanoglu et al. used 25 g per 10 minutes. Additionally, drug dosages were very different in these studies: 100 and 2.5 mg/kg. Fujimoto et al. concluded that melatonin has significant protective effects mainly on myelin sheaths, but also on the nucleus and mitochondria. Similarly, Kaptanoglu et al. suggested that melatonin not only has considerable protective effect against oxidative damage, but also reduces neutrophil-induced toxicity. A variety of SCI models has been used in experiments; the clip compression model has advantages over the weight drop model. The implementation of anterior and lateral spinal cord compression simulated vertebra corpus fractures in humans \((\text{Fujimoto et al., 2000; Kaptanoglu et al., 2000})\).

This study reveals that melatonin \((100 \text{ mg/kg})\) is an effective therapy for treating SCI. The results demonstrated that the use of melatonin for treating SCI decreased water content, aquaporin-4 and GFAP levels in the spine.

SCI leads to disability, sensory disorder and even death \((\text{Samantaray et al., 2008})\). To minimize or prevent these outcomes, we need to understand the pathophysiology of acute SCI to find effective therapeutic interventions. SCI starts with mechanical compression of the spinal cord. The spinal cord will present dropsy, microvascular permeability changes, and altered blood flow \((\text{Rowland et al., 2008; Tator et al., 1991})\). Traumatic injury of the spinal cord will always induce edema, mainly in the gray matter of the spinal cord \((\text{Shepard et al., 1999; Oshio et al., 2004})\). Subsequent pathophysiological injuries cause apoptosis \((\text{Ray et al., 2003})\). The active and progressive damage will soon spread and will continue for some time after the initial injury \((\text{Park et al., 2010})\). The present study in rats examined the protective effects of melatonin \((100 \text{ mg/kg})\) on treating edema from 12 to 72 hours after SCI.

The water content of the spinal cord invariably increases after SCI but it could be reduced significantly after 24 and 72 hours after injury by early administration of melatonin \((100 \text{ mg/kg})\). However, the effects of melatonin were not seen at 12 hours after injury. Our results demonstrated that long-term use of melatonin could decrease the spinal cord edema. As we described previously, the pathophysiology of SCI could be classified into primary or secondary damage. Primary damage consists of microvascular bleeding, which can increase water content in the spinal cord. The spinal cord water content at 12 hours after SCI may be affected by both primary and secondary damages, but the treatment with melatonin cannot mitigate the primary damage. The action of melatonin needs further investigation to optimize its therapeutic value.

Immunohistochemistry and western blot assay were used to study aquaporin-4 and GFAP protein levels after SCI. Aquaporin-4 \((100 \text{ mg/kg})\) was effective in reducing edema. Our results showed that melatonin \((100 \text{ mg/kg})\) can reduce edema. Our results showed that melatonin \((100 \text{ mg/kg})\) has a beneficial, anti-edema effect from 24 to 72 hours after SCI and that the level of aquaporin-4 decreases, which suggests that a lower level of aquaporin-4 expression may be linked to the decrease in spinal cord edema in rats.

Cellular swelling is significant after the central nervous system is injured and astrocytic swelling was more apparent than neuronal swelling \((\text{Marmarou et al., 2014})\). Our results showed that melatonin \((100 \text{ mg/kg})\) reduced GFAP protein levels in the spinal cord 2–3 days after SCI. Melatonin effectively removes astrocytic swelling caused by SCI. In short, in the injured spinal cord segments, the expression of aquaporin-4 and GFAP increases. Melatonin \((100 \text{ mg/kg})\) administered after SCI only reduces aquaporin-4 and GFAP levels in spinal cord segments between 24 and 72 hours after SCI. The results of this experiment show that melatonin \((100 \text{ mg/kg})\) can ameliorate edema after SCI. The lowered edema may reduce the level of expression of aquaporin-4. The reduction in the expression of GFAP may also help melatonin \((100 \text{ mg/kg})\) to reduce astrocytic swelling after SCI. This study of melatonin has shown worthwhile benefits, but the effects of melatonin in SCI patients will require further investigations.

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