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Original Article

Melatonin attenuates early brain injury after subarachnoid hemorrhage by the JAK-STAT signaling pathway

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Abstract: Increasing studies have demonstrated the neuroprotective effect of melatonin in central nervous system (CNS) diseases. However, the potential application of melatonin in therapy of subarachnoid hemorrhage (SAH) is still unclear. This study explored the potential effect of melatonin on early brain injury (EBI) induced by SAH and investigated the underlying mechanisms. Adult rats were subjected to SAH. Melatonin or vehicle was injected intraperitoneally 2 hr after SAH. The mortality, SAH grade, neurologic score, brain water content, and neuronal apoptosis were evaluated. To explore further mechanisms, changes in JAK1/STAT3 signaling pathway and the levels of apoptosis-associated proteins were also examined. The results suggest that melatonin improved the neurologic deficits and reduced the brain water content and neuronal apoptosis. In addition, The JAK1 inhibitor, Ruxolitinib, was applied to manipulate the proposed pathway. Mortality, neurological scores, brain edema, cell apoptosis, and the expression of JAK1, STAT3, and cleaved caspase-3 proteins were assayed after 24 h SAH. Melatonin significantly improved neurological function and reduced neuronal apoptosis and brain edema at 24 h after SAH. The level of JAK1 was markedly up-regulated. Additionally, the level of cleaved caspase-3 was decreased by melatonin treatment. The beneficial effects of melatonin in SAH rats were partially suppressed by Ruxolitinib. In summary, our results demonstrate that melatonin treatment attenuates EBI following SAH via the JAK1/STAT3 signaling pathway.

Keywords: Melatonin, subarachnoid hemorrhage, early brain injury, cell cycle, JAK1/STAT3 signaling

Introduction

Subarachnoid hemorrhage (SAH) is a medical emergency associated with high mortality and morbidity. Despite major advances in surgical technique, radiology, and anesthesiology, the high rates of mortality and morbidity after SAH have not changed in recent years [1]. Early brain injury (EBI) usually occurs within 72 h after SAH and accounts for 60% of deaths. Because EBI is a major leading cause of poor outcomes for SAH patients [2], effective treatment against EBI has become a major goal in SAH patient care. The underlying mechanisms of EBI include blood-brain barrier (BBB) disruption, cerebral edema, elevation of intracranial pressure, reduction of cerebral blood flow, inflammation, and neuronal apoptosis [3-6]. Therefore, therapies that alleviate EBI may provide better outcomes in patients with SAH.

Melatonin (Mel, N-acetyl-5-methoxytryptamine) is primarily secreted by the pineal gland and is responsible for regulating circadian rhythms [7]. Numerous studies have also linked melatonin to anti-inflammatory and anti-apoptotic effects [8]. Melatonin is beneficial for several nervous system conditions, including neurodegenerative diseases [9, 10], cognitive disorders, learning and memory impairments, and anxiety disorders [11]. Importantly, melatonin also attenuates hemorrhagic injury under different experimental conditions in various organs, including the brain [12]. Previous studies have shown that neuronal apoptosis is observed in experimental SAH and in clinical

patients after SAH [13]. Furthermore, the severity of neuronal apoptosis is indirectly correlated with neurological function, which suggests that apoptosis may be a potential therapeutic target against EBI after SAH [14]. Recent studies have demonstrated that Mel is protective in an experimental SAH model demonstrated that Mel decreases mortality following severe subarachnoid hemorrhage [15]. However, knowledge regarding the molecular mechanisms underlying the protective effects of melatonin against SAH is still fragmentary.

In the current work, we hypothesized that melatonin may reduce brain edema formation and subsequent apoptosis, with the goal of reducing EBI after SAH, which might involve the JAK1/STAT3 signaling pathway.

Materials and methods

Animal preparation

Male Sprague-Dawley rats (250-300 g) were purchased from the animal center of Nanjing University (Nanjing, China). The rats were raised in a temperature-controlled room (23±2°C) with a standardized light/dark cycle (12 h/12 h), and free access to food and water. All experimental protocols were approved by the Animal Care and Use Committee of the Nanjing Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

SAH model

The pre-chiasmatic cistern SAH model was performed as described previously [16]. Briefly, the amount of 0.3 mL non-heparinized fresh autologous arterial blood from the femoral artery was slowly injected into the pre-chiasmatic cistern in 20 s with a syringe pump under aseptic technique. Animals in the sham group were injected with 0.3 mL saline. After operation procedures, the rats were then returned to their cages, and food and water were kept easily accessible. Two milliliters of saline were injected subcutaneously right after the operation. Heart rates and rectal temperature were monitored, and the rectal temperature was kept at 37°C±0.5°C by using a warm pad when required throughout the experiments. Herein, the brain tissue adjacent to the clotted blood was taken for analysis in our study.

Reagents

Mel, dimethyl sulfoxide (DMSO), pentobarbital sodium, and 4',6-diamino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal antibody against JAK1, rabbit polyclonal antibody against STAT3, Rabbit monoclonal antibody against cleaved caspase-3, and rabbit monoclonal antibody against β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA).

Mortality and neurological assessment

The neurological scores were measured in a blinded manner at 12 h, 24 h, 48 h, 72 h, and 120 h post-SAH, based on the Garcia scoring system with modifications. Animals were given a score of 3 to 21 in 1-number steps (higher scores indicate greater function). The minimum neurological score was 3, and the maximum was 21. Mortality was calculated at 6 h, 24 h, 48 h, 72 h, and 120 h after SAH. The observer had no knowledge of the treatment the rats had been administered. All scoring procedures were performed in a blinded manner.

TUNEL staining

Based on our previous study, TUNEL assay was performed to evaluate the apoptosis in the brain cortex. Briefly, 50 μL TUNEL reaction mixture was added on each sample, and the slides were incubated in humidified atmosphere for 60 min at 37°C in the dark. To detect the nuclei, the slides were incubated with DAPI for 5 min at room temperature in the dark. The slides were observed with a fluorescence microscopy. Apoptotic index was determined as the ratio of the number of TUNEL positive neurons to the total number of neurons.

Western blot

Western blot was performed as described previously. The left basal cortical sample was collected, and protein extractions were obtained. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon nitrocellulose membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% non-fat milk in TBST at room temperature and then incubated with antibodies against JAK1, STAT3, caspase-3, and β-actin (1:1000)

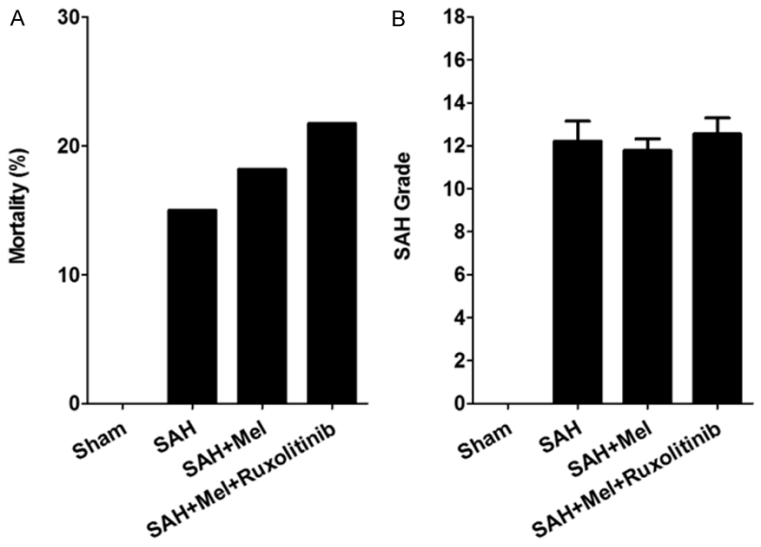

Figure 1. Mortality and SAH grade in each group (A and B). The SAH group mortality rate was 15% (3 of 20 mice), the SAH+Mel group rate was 18.18% (4 of 22 mice) and the SAH+Mel+Ruxolitinib group rate was 21.74% (5 of 23 mice). The SAH grade scores showed no significant difference between the SAH group, the SAH+Mel group, and the SAH+Mel+Ruxolitinib group. Mel melatonin, SAH subarachnoid hemorrhag.

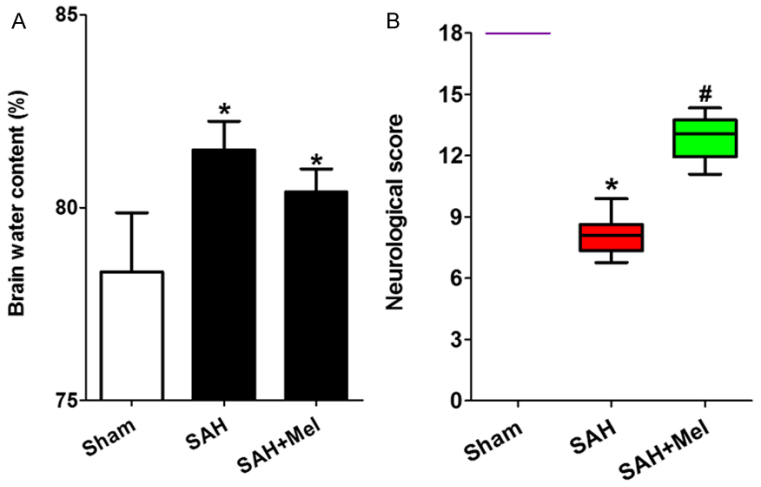

Figure 2. The effects of melatonin on brain water content and neurological score 24 h after SAH. Brain water content and neurological score of rats increased at 24 after SAH compared with that of rats in the sham group. Melatonin treatment dramatically decreased neurological score and alleviated brain edema at 24 h after SAH. The data are presented as the mean ± SEM, n=6 for each group. *P < 0.05 versus sham group, #P < 0.05 versus SAH group.

overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) at room temperature for 1.5 h. The protein density was detected using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using the Imagelab software package (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Graph Pad Prism6 and SPSS 18.0 were used to analyze data in this study. Data for SAH grades and neurological scores are expressed as median and 25th to 75th percentiles and were analyzed by the Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Dunn's or Tukey post hoc analysis. Other data are presented as the mean ± SEM. Fisher's exact test was used for mortality analysis. Multiple group comparisons were tested by one-way AN-|OVA. Tukey post hoc analysis was used for intergroup comparisons. $P < 0.05$ was considered significant.

Results

General observations and mortality rate

The mortality in each group was evaluated (**Figure 1A**). No rats died in the sham group. No statistical significance was observed for mortality between surgery groups. The SAH group mortality rate was 20% (7 of 35 rats), the SAH+Mel group rate was 18% (6 of 34 rats), and the SAH+Mel+Ruxolitinib group rate was 18% (6 of 34 rats).

Next, the SAH grade in each group was evaluated (**Figure 1B**). No significant difference was observed for mortality among the operated groups. When rats were sacrificed, subarachnoid blood clots were found on the inferior basal temporal lobes of all rats with experimentally induced SAH groups. Therefore, temporal lobe brain tissue was collected for analysis in this study.

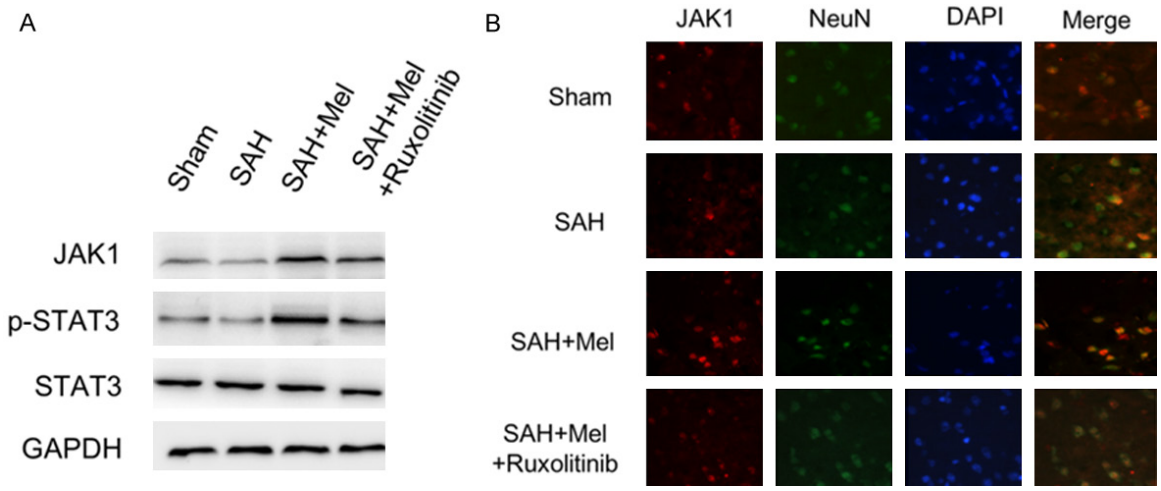

Figure 3. Effects of melatonin on JAK1/STAT3 signaling pathway. Western blot analysis of JAK1 and p-STAT3 (A) in the cortex after 24 h of SAH. The JAK1 and p-STAT3 protein level were significantly up-regulated by Mel treatment compared with the SAH and SAH+Mel+Ruxolitinib groups, which were down-regulated by ruxolitinib. Representative photomicrographs showed brain cortex double immunofluorescent staining for JAK1 (red) and NeuN (green) at 24 h after SAH (B). JAK1-positive cells colocalized mainly with neurons in the cortex. DAPI (blue) as a nuclear marker.

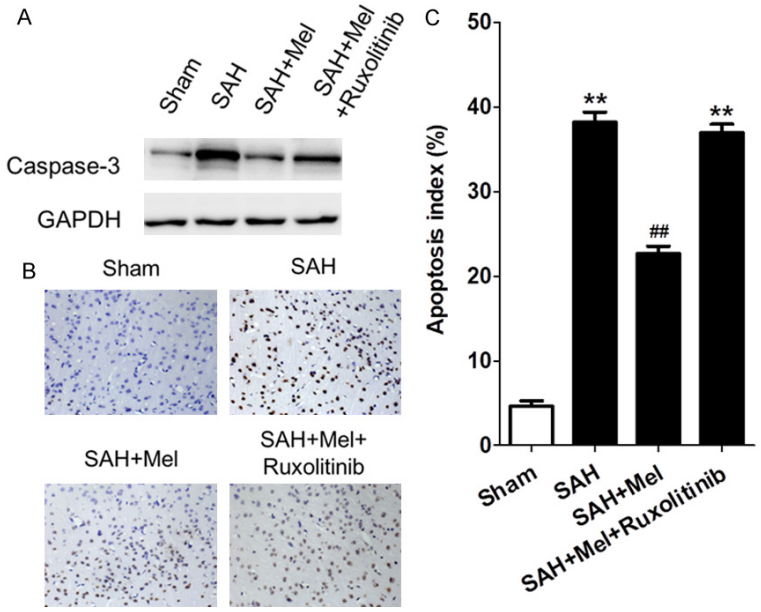

Figure 4. Effect of ghrelin treatment on neuronal apoptosis 24 h after SAH. Western blot analysis of caspase-3 (A) in the cortex after 24 h of SAH. The expression of caspase-3 protein in the SAH+Mel group was markedly decreased compared with the SAH group, and this was inhibited by ruxolitinib administration. A high apoptotic index was found in the SAH group compared to the control group (B). However, compared with the SAH group, the apoptotic index in the SAH+Mel+Ruxolitinib group was significantly decreased (C), which was reversed by ruxolitinib.

Melatonin alleviated the neurological outcomes following SAH

Twenty-four hours after SAH, brain water content and neurological scores were measured. The rats in the SAH group showed an increase in brain water content and a decrease in neurological score. Mel administration decreased the brain water content and improved the neurological deficits at 24 h following SAH in the SAH+Mel group (vs. the SAH group, $P < 0.05$) (**Figure 2A** and **2B**).

Effects of melatonin on JAK1/STAT3 signaling pathway

Western blot analysis revealed a significant up-regulation of JAK1 in the SAH+Mel group compared with the control group, SAH group and SAH+vehicle group, and there was no significant difference among the control group, SAH group and SAH+Mel group in brain JAK1 protein level (**Figure 3A**). The JAK1 level of rats in the group was significantly lower than that of rats in the SAH+Mel group (**Figure 3A**). To identify in which kind of brain cells JAK1 was predominantly expressed after SAH, double immunofluorescence staining was performed for JAK1 and neuron-specific nuclear protein. We found that the JAK1-positive cells

colocalized mainly with NeuN-positive neurons (**Figure 3B**), which suggested that JAK1 was induced mainly in neurons. These results suggest that the expression of JAK1 is related to neuronal cell survival after SAH. Consistent with the western blot results, enhanced accumulation of JAK1 was observed in the SAH+Mel group, and low JAK1-positive staining was detected among the SAH, SAH+Mel and SAH+Mel+Ruxolitinib groups.

Effect of melatonin on caspase-3 expression and neuronal apoptosis after SAH

Western blot analysis was performed to assess the level of cleaved caspase-3 expression. The level of cleaved caspase-3 in the SAH group and the SAH+Mel group was significantly higher than that of the control group ($P < 0.01$; **Figure 4A**). After Mel treatment, the cleaved caspase-3 protein level was significantly decreased. This decrease in the cleaved caspase-3 level could be inhibited by Ruxolitinib-there was a significant difference between the SAH+Mel group and SAH+Mel+Ruxolitinib group ($P < 0.05$; **Figure 4A**).

We observed widespread distribution of TUNEL-positive cells in the temporal lobe region in the SAH group and SAH+Mel group, and the apoptotic index was increased significantly compared with that of the control group ($P < 0.01$; **Figure 4B**). Mel administration in the SAH rats significantly decreased neuronal apoptosis as demonstrated by the low apoptotic index in the SAH+Mel ($P < 0.01$; **Figure 4C**). Co-treatment with Ruxolitinib inhibited the anti-apoptotic effect of Mel. The apoptotic index of the SAH+Mel+Ruxolitinib group was comparable to that of the SAH group.

Discussion

The major findings of the present study are as follows: (1) Mel protects the brain from EBI following SAH; (2) Mel attenuates cerebral edema and neural inflammation and improves neurological score in mice subjected to SAH; (3) JAK1/STAT3 signaling is involved in the protective effect of Mel. In the present study, Mel administration after SAH significantly improved the neurological score. In addition, 24 h after SHA, Mel administration attenuated cerebral edema and neuronal apoptosis.

Mel, a circadian hormone primarily secreted by the pineal gland, has been reported to exhibit various pharmacological properties. Mel is well known for the regulation of the circadian rhythm in human beings and known for its protective effects against various disorders. A recent study suggests that Mel prevents kidney injury in a high-salt diet-induced hypertension model by decreasing oxidative stress [17]. As for the neurological disorders, Mel has been reported to prevent neural tube defects in the offspring of diabetic pregnancy [18]. Moreover, Mel protects against cerebral ischemia via Sirt1 activation [19]. The protective effects of Mel against SAH have also been investigated. Chen et al. demonstrated that Mel attenuates inflammatory response-induced brain edema in early brain injury following SAH [12]. Moreover, Mel attenuates EBI following SAH by enhancing autophagy and activating the Nrf2-ARE pathway [20, 21]. In the present study, our results demonstrate that Mel attenuates EBI following SAH via JAK1/STAT3 signaling pathway.

As a signaling pathway that regulates cell survival, the JAK1/STAT3 pathway has been implicated in many diseases including several nervous system diseases [22]. Inflammation in response to SAH may play an important role in vasospasm and a prior study showed that the JAK-STAT pathway is activated in basilar arteries after SAH. JAK-STAT signaling appears to play an important cardioprotective role in ischemic preconditioning through upregulation of COX-2. Expression of COX-2 in the endothelial cells through JAK-STAT signaling pathway was upregulated from 6 h after onset of SAH. The induced COX-2 plays vasodilator effects in antagonizing delayed cerebral vasospasm after SAH, which occurs maximumally at day 2 in this single-hemorrhage model [23]. In the present study, Mel treatment activates JAK1 and promotes the phosphorylation of STAT3. JAK1 antagonist abolishes this effect, indicating that JAK activation is involved in the protective effects of Mel against EBI following SAH.

In summary, our investigation provides new insights into the mechanisms underlying Mel's protective effects against EBI following SAH. The present study suggests that Mel improves neurological score, attenuates brain edema and neuronal apoptosis, and increases JAK1 expression and decreases caspase-3 expression via the JAK-STAT signaling pathway.

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Disclosure of conflict of interest

None.

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