



Melatonin Attenuates White Matter Injury via Reducing Oligodendrocyte Apoptosis After Subarachnoid Hemorrhage in Mice

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ABSTRACT

AIM: To determine whether melatonin (MLT) mitigates white matter (WM) injury by attenuating NOD-like receptor family pyrin domain-containing 3 (NLRP3)-associated oligodendrocyte apoptosis after subarachnoid hemorrhage (SAH).

MATERIAL and METHODS: SAH model C57BL/6J mice were created using an endovascular perforation technique. The mice were injected intraperitoneally with MLT at doses of 50 mg/kg, 150 mg/kg and 300 mg/kg. The animals were evaluated for neurological outcomes according to neurological score, brain water content, myelin degradation, amyloid precursor protein (APP) accumulation, apoptosis, and levels of NLRP3, caspase-1, interleukin-1 β , Bcl-2, and Bcl-2-interacting mediator of cell death (Bim) expression after SAH.

RESULTS: MLT at a dose of 50 mg/kg improved neurological score, alleviated brain edema, and reduced WM injury. In addition, loss of myelin basic protein (MBP) and accumulation of APP, and expression in the ipsilateral/left hemisphere were found after SAH, and were reversed by MLT treatment. NLRP3 signal activation was found in microglia and apoptosis in oligodendrocytes were observed after SAH. MLT reduced oligodendrocyte apoptosis by regulating Bim and Bcl-2.

CONCLUSION: Results of this study indicated that MLT exerts a WM-protective effect in SAH pathophysiology, possibly by attenuating apoptosis in oligodendrocytes.

KEYWORDS: Subarachnoid hemorrhage, White matter, Oligodendrocyte, Melatonin, Apoptosis, Mice

ABBREVIATIONS: ANOVA: Analysis of variance, **APP:** Amyloid precursor protein, **BBB:** Blood-brain barrier, **Bcl-2:** B cell leukemia/lymphoma 2, **Bim:** Bcl-2-interacting mediator of cell death, **EBI:** Early brain injury, **IL-1 β :** Interleukin-1 β , **MBP:** Myelin basic protein, **MLT:** Melatonin, **PBS:** Phosphate-buffered saline, **SAH:** Subarachnoid hemorrhage, **WM:** White matter.

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INTRODUCTION

Subarachnoid hemorrhage (SAH) is commonly encountered in the clinic. It is usually caused by rupture of an aneurysm and is associated with high morbidity and mortality (20). A recent theory posited that early brain injury (EBI) is the most promising target for treatment of SAH. EBI includes neuronal death, surrounding apoptosis, microcirculation dysfunction, disruption of the blood brain barrier (BBB), brain edema, and other pathophysiological mechanisms in the early stages of SAH (23). Approximately 50% of tissue in the human brain is white matter (WM), which contains neuronal axons and oligodendrocytes. However, acute WM injury has not been extensively studied in experimental models of SAH. Several clinical and experimental studies have indicated that WM may be involved in neurological outcomes after SAH (7,11,16,18). Therefore, to alleviate WM injury after SAH and improve neurological deficits, it is critical to develop novel therapies.

Previous investigations have indicated that inflammation and apoptosis appear early and play a critical role in neurological deficits after SAH (2). NOD-like receptor family pyrin domain-containing 3 (NLRP3) has been considered to be a key factor in both bacterial and aseptic inflammation during pathophysiological processes, and involves activated downstream caspase-1/IL-1 β (25). Melatonin (MLT) has recently been found to protect and prevent neurological deterioration by reducing inflammation in preclinical experiments. Because MLT is amphipathic and easily passes through the BBB, we deliberated whether MLT could affect WM injury and attenuate EBI. The present study aimed to investigate the role of MLT in neurological impairment and pathophysiological changes in WM using an experimental mouse model of SAH. We hypothesized that MLT attenuates WM injury by preventing NLRP3-associated microglia activation and oligodendrocyte apoptosis. Our study aimed to produce robust and reliable data to support this hypothesis.

MATERIAL and METHODS

Mice

Animal operations were approved by the Ethic Committee of General Hospital of Northern Theater Command (General Hospital of Shenyang Military Command), and the procedures complied with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Decision date: Nov/1st/2017; Number: 2017024). In this study, 108 male C57BL/6J mice, weighing 22 g to 30 g (purchased from the Department of Experimental Animal of General Hospital of Shenyang Military Command), were randomly divided into 6 groups as follows: sham (n=24); SAH (n=12), SAH + vehicle (n=24); SAH + MLT 50 mg/kg (n=12); SAH + MLT 150 mg/kg (n=24); and SAH + MLT 300 mg/kg (n=12).

SAH Model

Murine SAH model were created using an endovascular perforation technique, similar to a method previously described (30). Briefly, mice were anesthetized by intraperitoneal (i.p.)

injection of sodium pentobarbital (40 mg/kg), followed by exposure of the bifurcation of the common carotid artery using blunt dissection. The external carotid artery (ECA) was sheared distally to leave a stump, if possible. A 5-0 sharpened monofilament nylon suture was inserted from the stump of the ECA directly to the internal carotid artery (ICA) and pierced the intracranial bifurcation of the ICA terminal segment. During the surgical procedure, an electric blanket was placed under the animals maintain rectal temperature at 37°C. Mice in the sham group underwent all of the same procedures but without perforation.

The extent of hemorrhage in the subarachnoid space was evaluated according to a previously described grading system (24). Cistern on the ventral surface of the brain was partitioned into 6 parts, each of which was scored from 0–3 for the level of blood clot. The total score was the sum of scores for the 6 areas (0–18 points). Exclusions were made when the total score was < 8 without remarkable neurological impairment.

Assessment of Neurological Deficit

Neurological scores were obtained using a protocol incorporating the modified Garcia score and beam balance score at 24 hours (h) (n=6) and 72 h (n=6) after SAH. These two scales have been previously described and validated (21). Two blinded observers evaluated mean neurological scores.

Drug

MLT (Sigma-Aldrich, Shanghai, China) 50 mg/kg, 150 mg/kg, 300 mg/kg, and vehicle (1% alcohol) were intraperitoneally injected 15 min after induction of SAH. Mice in the sham group were not injected.

Brain Water Content

Mice were euthanized 24 h and 72 h after SAH, and brain specimens (n=6) were carefully dissected away from the skull and quickly cut into bilateral hemispheres, cerebellum, and brain stem. The four separate parts were weighed on a small sheet of aluminum foil to measure wet weight (4). The four parts were dried at 57°C for 48 h to measure dry weight. Brain water content was calculated using the following equation:

$$\text{Water content} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%.$$

Terminal Deoxynucleotidyl Transferase dUTP Nick End-labeling Staining

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed as previously described (3). Brain sections (n=6) were stained using a commercially available kit according to manufacturers' recommendations (Roche, Mannheim, Germany). Briefly, slides were immersed in proteinase-K in Tris-HCl (10 mM) buffer at 37 °C for 15 min, then rinsed with phosphate-buffered saline (PBS) and incubated in 0.3% hydrogen peroxide. The slides were then immersed in sodium citrate (0.1%) and Triton X-100 (0.1%) solution for 3 minutes. After 3 washes with PBS, the sections were incubated with the TUNEL reaction mixture, containing TdT and Cy3-dUTP, for 60 min at 37°C.

Immunohistochemistry

Fixed, frozen brain sections were subjected to immunofluorescence staining (n=6) as previously described using the following primary antibodies: anti-MBP (1:500 dilution; Santa Cruz Biotechnology, TX, USA), anti-Iba-1, anti-amyloid precursor protein (APP), anti-interleukin (IL)-1 β , anti-CNPase, and anti-NeuN (1:500 dilution; Abcam, Cambridge, United Kingdom) (9). The slides were then examined using a fluorescence microscope (Zeiss, Oberkochen, Germany). Myelin basic protein (MBP) and APP per field were counted using ImageJ software. Brain sections were stained with Luxol fast blue (LFB) for myelin imaging (n=1) in accordance with a previously described method (8).

Western Blot

The protocol used for Western blotting was similar to that described in the authors' previous work (32). Briefly, the corpus callosum and the internal capsule in the left hemisphere (n=6) were isolated macroscopically. Proteins were detected using the following primary antibodies: anti-caspase-1, anti-NLRP3, anti-IL-1 β (1:1000, Cell Signaling Technology, Boston, MA, USA), anti-Bcl-2, anti-Bcl-2-interacting mediator of cell death (Bim) (1:500; Abcam, Cambridge, United Kingdom). Anti- β -actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control.

Statistical Analysis

Data are expressed as mean \pm standard error of the mean (SEM). Comparison among the groups was performed using one-way analysis of variance (ANOVA) and the Tukey post hoc multiple comparisons test. All statistical evaluations were performed using GraphPad Prism version 6 (GraphPad Inc, San Diego, CA, USA); differences with $p < 0.05$ were considered to be statistically significant.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Ethics Committee of General Hospital of Northern Theater Command (General Hospital of Shenyang Military Command) and performed in accordance with the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and adhered to the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments, <https://www.nc3rs.org.uk/arrive-guidelines>).

RESULTS

MLT Improved Neurological Function After SAH

No mice in the sham group died; however, 1 in the SAH group, 3 in the SAH + vehicle group, and 2 in the SAH + MLT group (1 in the SAH+MLT 50 mg/kg group, 3 in the SAH + MLT 150 mg/kg group, and 1 in the SAH + MLT 300 mg/kg group) died within 24 h after SAH on account of mass clot in the subarachnoid space. There were no significant differences in grading scores among the groups at 24 h and 72 h (Figure 1A, E).

To investigate whether MLT attenuated neurological deficits, mice in SAH and SAH + vehicle groups were evaluated using

the modified Garcia and beam balance tests. At 24 h and 72 h after SAH, modified Garcia scores were clearly lower in the SAH and SAH + vehicle groups compared with mice in the sham group (Figure 1C, G). Neurological deficits were attenuated by MLT treatment at doses of 50 mg/kg, 150 mg/kg, and 300 mg/kg (Figure 1C, G). Furthermore, the beam balance test also indicated significant neurological impairment in the SAH and SAH + vehicle groups, which was alleviated by MLT treatment (Figure 1B, F).

MLT Alleviated Brain Edema and WM Injury

Brain edema can independently indicate poor neurological outcome after SAH. To investigate whether brain edema developed after SAH, the protective effect of MLT on WM in the MLT- and vehicle-treated groups were compared. Data revealed higher brain water content in the vehicle group than in the sham group in both hemispheres (Figure 1D, H). In addition, treatment with MLT 50 mg/kg, 150 mg/kg, and 300 mg/kg effectively attenuated brain edema (Figure 1D, H). There was no significant difference among the 50 mg/kg, 150 mg/kg and 300 mg/kg doses. As such, a dose of 50 mg/kg was chosen for subsequent follow-up experiments.

Loss of myelin was found in the corpus callosum and internal capsule of the ipsilateral/left hemisphere in the SAH and SAH + vehicle groups at 24 h, and was attenuated by MLT treatment at a dose of 50 mg/kg (Figure 2A). WM structures contain axons and myelin sheath. Axonal accumulation of APP is a classic marker of axonal injury indicative of cytoskeletal damage (14, 18). Demyelination can be reflected by a downtrend of MBP, a primary component of the myelin sheath (19). Mice in the SAH + vehicle group exhibited apparent decreases in MBP and accumulation of APP in the left hemisphere 24 h after SAH. MLT treatment (50 mg/kg), resulted in loss of MBP and APP accumulation were attenuated compared with the vehicle group (Figure 2C-F). Moreover, mice in the vehicle group exhibited more TUNEL-positive (apoptotic) cells compared with sham-treated mice. In contrast, MLT treatment (50 mg/kg) eliminated apoptotic cells in WM areas (Figure 2G, H).

MLT Attenuated NLRP3-Induced Apoptosis of Oligodendrocytes

Apoptosis of neurons and oligodendrocytes induced by NLRP3 activation plays a vital role in the pathophysiology of SAH (10). Previously, the authors reported that MLT could suppress NLRP3 activation, which induces subsequent caspase-1 and IL-1 β maturation (5). Under pathological conditions in the central nervous system, microglia act as a main source of NLRP3 production. Microglia accumulation in the WM and increasing numbers of apoptotic oligodendrocytes were observed after SAH (Figure 3A-D). After MLT delivery, microglia accumulation and oligodendrocyte apoptosis were attenuated. Moreover, protein levels of NLRP3, caspase-1, and IL-1 β were elevated at 24 h post-SAH (Figure 4A-D) and MLT treatment reversed these alterations. Furthermore, Western blot also confirmed elevation of Bcl-2 and decreases in Bim (Figure 4E-G). These effects were reversed by treatment with MLT (50 mg/kg).

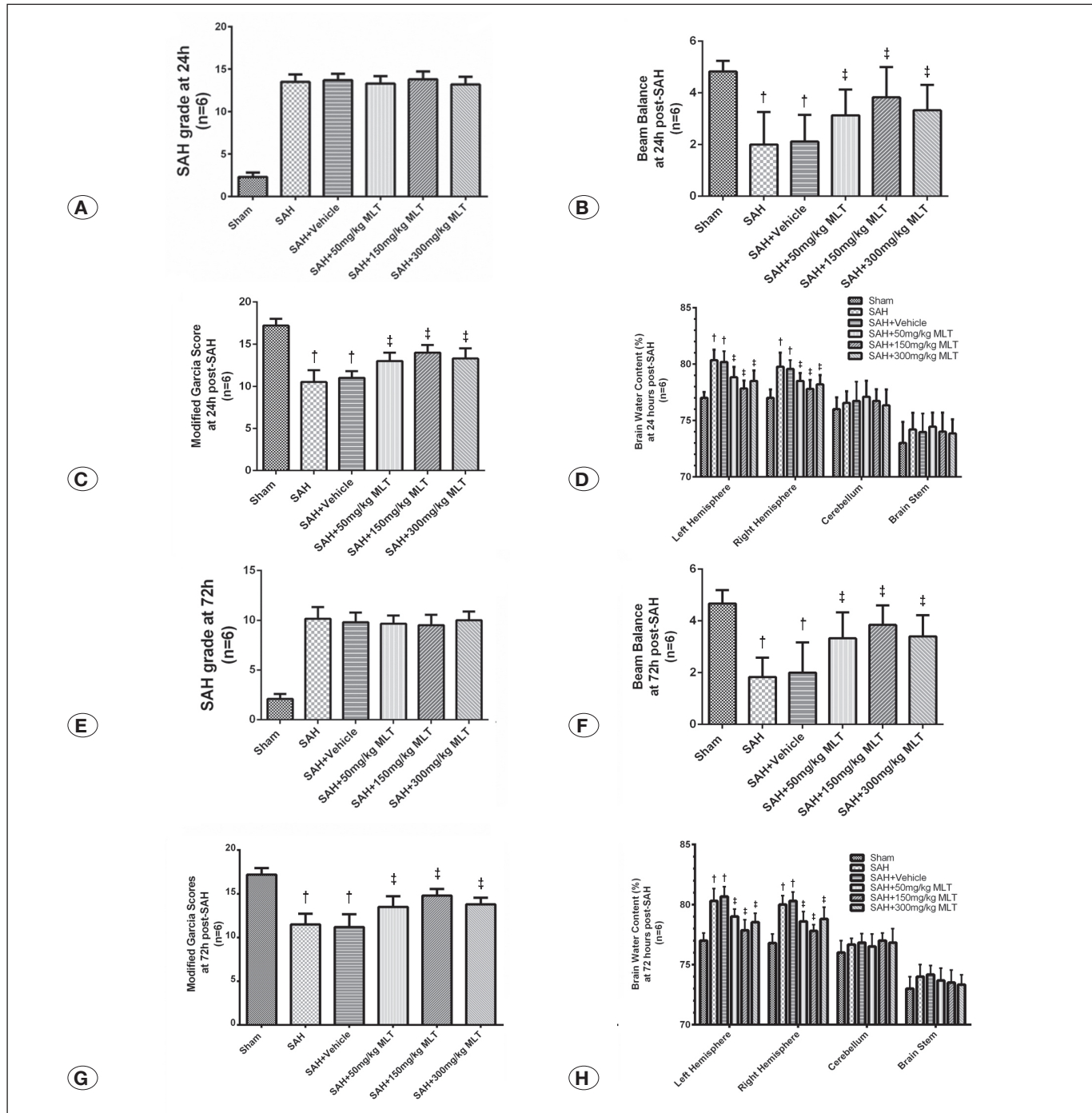


Figure 1: Neurological function assessment and brain edema by melatonin (MLT) treatment after subarachnoid hemorrhage (SAH). **A)** SAH grading scores revealed no significant differences among vehicle, MLT 50 mg/kg, MLT 150 mg/kg and MLT 300 mg/kg 24 hours (h) after SAH. **B, C)** Neurological tests showed significantly lower score in SAH and SAH + vehicle group compared with sham group. MLT 50 mg/kg, 150 mg/kg, and 300 mg/kg treatment could increase the behavior text score significantly. Modified Garcia scores and Beam balance text revealed no different among 50 mg/kg, 150 mg/kg, and 300 mg/kg. **D)** Brain edema was observed at 24 h after SAH. In contrast to SAH and SAH + vehicle groups, MLT 50 mg/kg, 150 mg/kg, 300 mg/kg administration reduced brain water content efficiently. **E)** SAH grading scores demonstrated no significant differences among vehicle and MLT (50 mg/kg, 150 mg/kg, and 300 mg/kg) 72 h after SAH. **F, G)** At 72 h behavior test results were profoundly lower in the SAH and SAH + vehicle groups after SAH induction and reversed by treatment with MLT at doses of 50 mg/kg, 150 mg/kg, and 300 mg/kg. **H)** At 72 h, brain edema was detected after SAH, but decreased by MLT treatment at doses of 50 mg/kg, 150 mg/kg, and 300 mg/kg compared with the SAH and SAH + vehicle groups. †Statistically significant difference (i.e., $p < 0.05$) compared with the sham group; ‡Statistically significant difference (i.e., $p < 0.05$) compared with sham group, SAH + vehicle ($p < 0.05$).

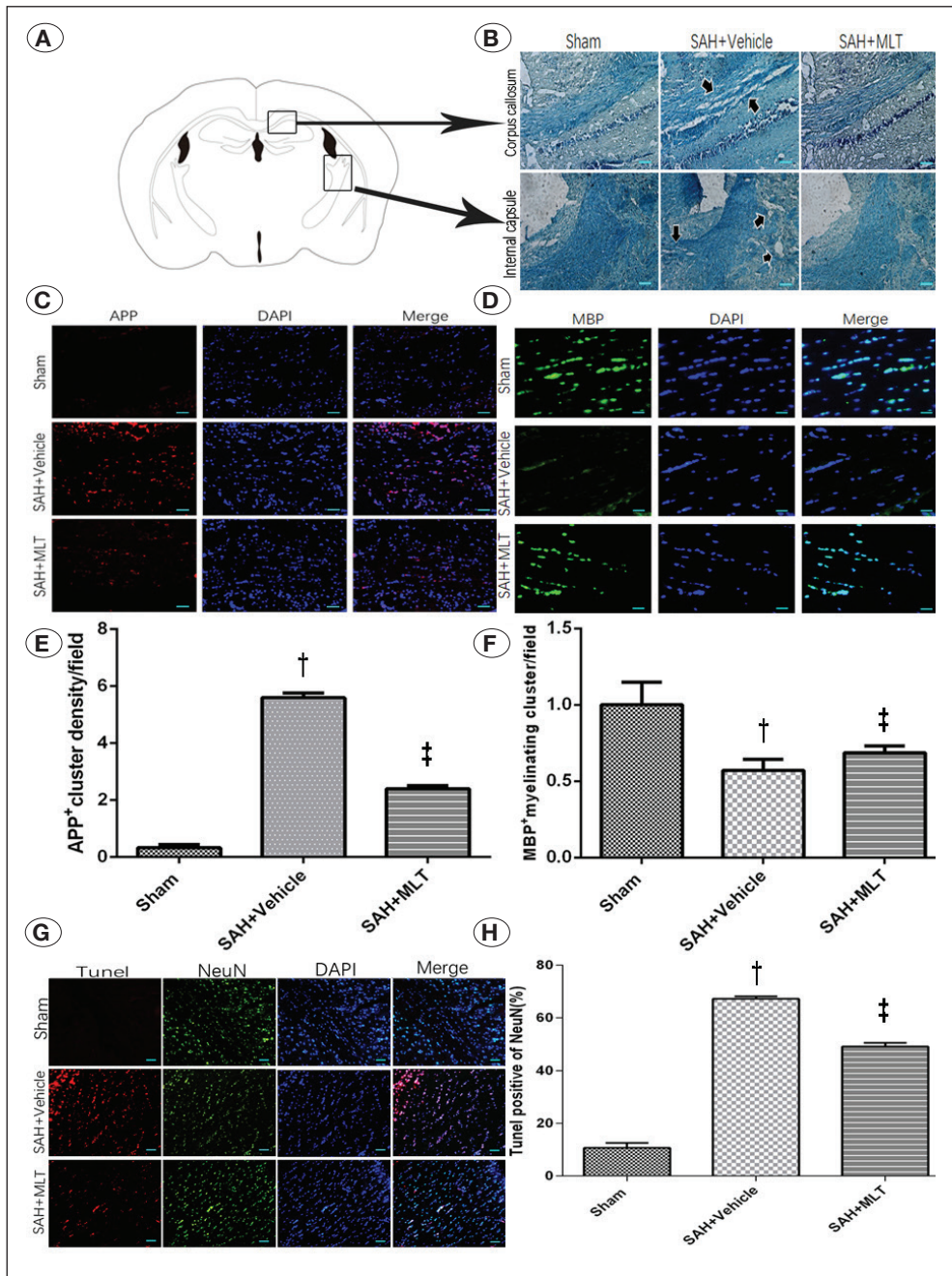


Figure 2: White matter (WM) injury and neuronal apoptosis after subarachnoid hemorrhage (SAH). **A)** Sketch map of brain coronary section and areas stained by Luxol fast blue (LFB). **B)** LFB staining demonstrating marked disturbances in the WM of the left hemisphere 24 hours (h) after SAH in vehicle-treated SAH mice. After MLT injection (50 mg/kg), the disturbance was apparently attenuated in the ipsilateral hemisphere. Scale bar = 20 μ m.

C-F) Immunofluorescence revealing loss of myelin basic protein (MBP) and accumulation of amyloid precursor protein (APP) 24 h after SAH in left WM area, which was reversed by MLT treatment. C, Scale bar = 20 μ m; D, Scale bar = 10 μ m.

G-H) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of neurons increasing by SAH induction and reduced by MLT treatment 24 h after SAH. G, Scale bar = 20 μ m.

[†]Statistically significant difference (i.e., $p < 0.05$) compared with sham group; [‡]Statistically significant difference (i.e., $p < 0.05$) compared with sham group; SAH + vehicle $p < 0.05$.

DISCUSSION

Results of the present study demonstrated improved neurological scores after treatment with MLT compared with vehicle in an experimental mouse model of SAH. We further demonstrated that MLT reduced brain edema and provided protection of the WM against acute injury in EBI. Additionally, these phenomena were associated with NLRP3-induced WM injury and neuronal apoptosis. As we demonstrated, MBP was downregulated and APP accumulated after SAH but were reversed by treatment with MLT (50 mg/kg). These findings suggest that MLT demonstrates promising potential in treating EBI after SAH.

Various pathophysiological changes, such as the mass effect of clot, increasing intracranial pressure, decompensation of cerebral autoregulation, spreading depolarization, BBB breakdown, release of toxic blood components, secondary inflammation, and cell death occur immediately after hemorrhage (13). These injuries contribute to poor neurological outcome(s); however, few studies have focused on WM injury after SAH (22). The effect of WM fiber injury on neurological deficits, consciousness, and memory after SAH may be overlooked due to the paucity of WM in the brains of rodents, which are the most common experimental animals (17). Results of a study using a mouse model of SAH suggested that the WM is particularly

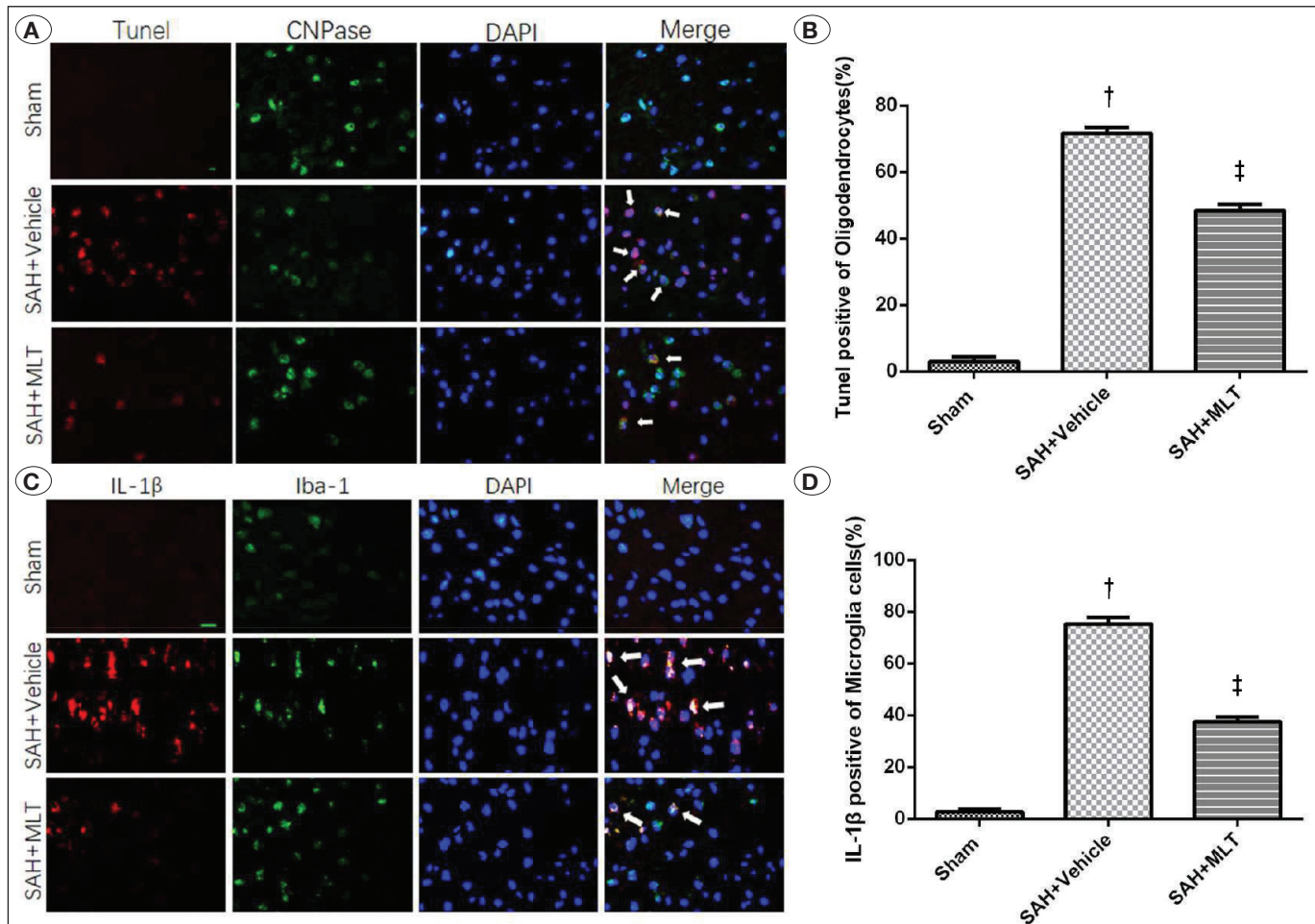


Figure 3: Protein levels of myelin basic protein (MBP), amyloid precursor protein (APP) accumulation, and expression of NOD-like receptor family pyrin domain-containing 3 (NLRP3) signal in the ipsilateral white matter. **A-B)** Representative Western blot images of MBP and APP demonstrating increasing APP and decreasing MBP in the subarachnoid hemorrhage (SAH) + vehicle group, and significant decreases after melatonin (MLT) treatment 24 hours (h) after SAH. **C-D)** NLRP3, caspase-1, and interleukin (IL)-1 β expression levels were elevated at 24 h. MLT treatment ameliorated these increasing trends. Density values of the blot were set to 1 (i.e., reference) in the sham group, and the other groups were compared with the sham group. [†]Statistically significant difference (i.e., $p < 0.05$) compared with sham group; [‡]Statistically significant difference (i.e., $p < 0.05$) compared with SAH + vehicle group.

vulnerable to injury by SAH. Diffusion tensor imaging (DTI) and histochemistry revealed typical pathological alterations including axonal injury in WM areas (18). Small-sample clinical experimental results suggested that DTI parameters, including fractional anisotropy and apparent diffusion coefficient, in WM areas are associated with SAH prognosis (11,12). Our results revealed APP accumulation and loss of MBP in the WM after SAH, which may have been due to NLRP3 activation and downstream caspase-1 and IL-1 β in microglia and the induction of apoptosis in oligodendrocytes. A dose of 150 mg/kg MLT inhibited NLRP3 expression, protected WM fibers, and improved neurological score, indicating that targeting WM protection may be potentially beneficial to patients who experience SAH.

MLT is a hormone produced by the pineal gland, which regulates sleep and wakefulness, and has been used for the treatment of insomnia in the clinic (26). Its amphipathic nature

enables MLT to cross the BBB. Our recent study revealed that MLT could inhibit the NLRP3 inflammasome after stroke (5), and other experimental studies indicated that MLT ameliorates WM injury after neurological insult (6,28). However, these data are still insufficient to explain how MLT regulates NLRP3 signal and protects WM. In the present study, we demonstrated that MLT inhibited the NLRP3 signal, as well as the downstream expression of caspase-1 and IL-1 β in microglia in areas of WM. Microglia are essential inflammatory cells in the central nervous system after SAH. A recent study reported that microglia accumulate in the WM after SAH; however, its acute impact remains elusive (29). Our data demonstrated NLRP3 signal activation in microglia and microglia accumulation in the WM post-SAH. MLT treatment alleviated oligodendrocyte apoptosis by altering Bim and Bcl-2. Future time-effect investigations may provide more evidence supporting the utility of MLT in clinical conditions.

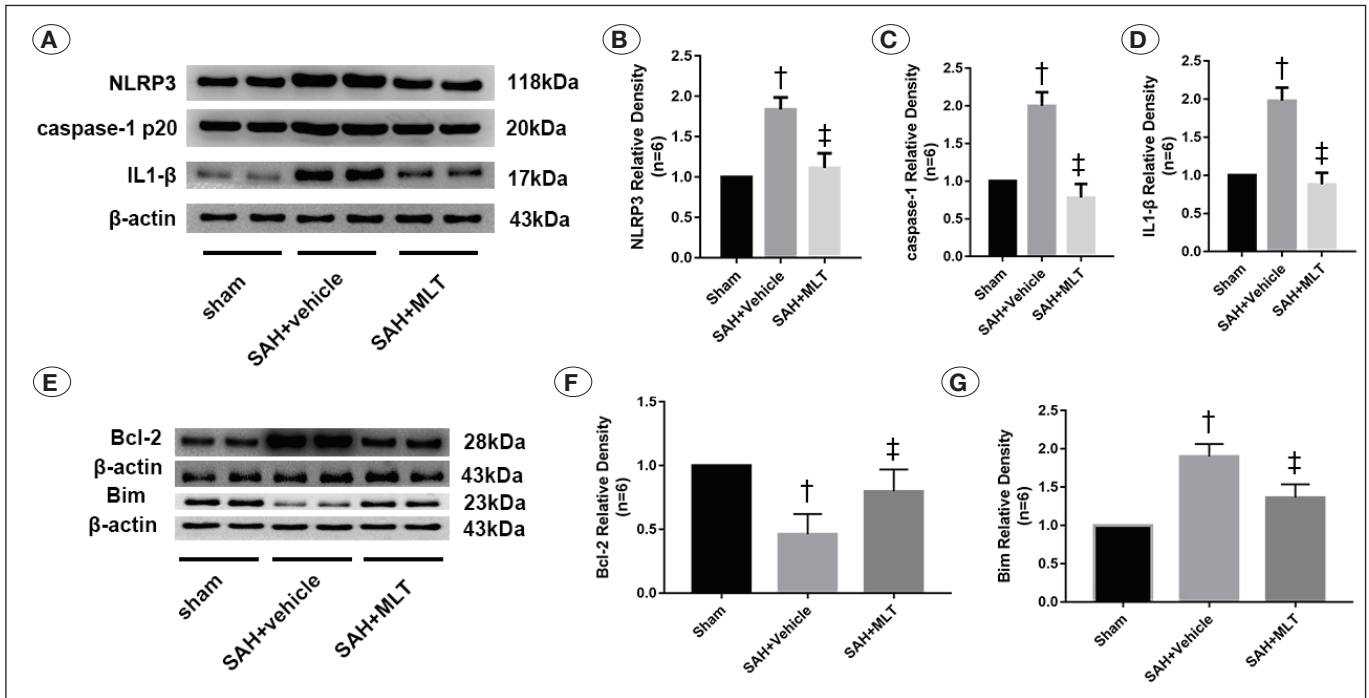


Figure 4: Protein levels of NOD-like receptor family pyrin domain-containing 3 (NLRP3) and apoptosis-related proteins in the ipsilateral white matter. **A-D)** Protein expression levels of NLRP3, caspase-1, and interleukin (IL)-1 β are increased in mice in the subarachnoid hemorrhage (SAH) + vehicle group and reduced by melatonin (MLT) treatment 24 h after SAH. **E-G)** Protein expression levels of Bcl-2 and Bcl-2-interacting mediator of cell death (Bim). Bcl-2 expression levels increased while Bim levels were reduced 24 h after induction of SAH. MLT treatment effectively reversed these trends. [†]Versus sham group ($p < 0.05$); ^{*}Versus SAH + vehicle ($p < 0.05$).

Except for roles in anti-inflammation, MLT has also been reported to reduce neurological deficits in other conditions, such as acting as an anti-oxidant (1). MLT pretreatment profoundly reduced ischemia/reperfusion-induced autophagic flux in a mouse model of ischemic stroke. Furthermore, MLT ameliorated ischemia/reperfusion-induced neuronal apoptosis via the PERK/ATF4/CHOP pathway in endoplasmic reticulum stress (9). Recently, NLRP3 inflammasomes, which are critical for the formation of local inflammatory lesions, were found to be correlated with WM injury after induction of experimental multiple sclerosis (10). In a cuprizone model of demyelination, MLT dose-dependently decreased the mean number of apoptotic cells via caspase-3, Bax and Bcl-2 apoptotic signaling (27). These phenomena may be inhibited NEK-7 NLRP3 interaction and downstream inflammation activation (15,31); nevertheless, further investigation should be conducted.

The present study had some limitations. Regarding the time course of drug administration, we investigated a one-time injection of MLT after SAH. A time-effect curve should be investigated in future basic studies. Regarding mechanism, we did not completely exclude other underlying molecular mechanisms associated with the protective effects of MLT on WM. Third, the upstream mechanism in which MLT influences NLRP3 signal, MBP, and APP after SAH remain incompletely elucidated. Therefore, more studies are needed to explain the exact mechanisms of WM protection by MLT before clinical use.

CONCLUSION

Results of the present study demonstrated that MLT attenuated EBI after SAH via NLRP3-induced apoptosis of oligodendrocytes. MLT exhibited the ability to cross the BBB in the central nervous system and has already been used in the clinic; therefore, it may be potentially beneficial to individuals who experience SAH.

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