

Research Article

Melatonin attenuates microglial activation and improves neurological functions in rat model of collagenase-induced intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) is a severe form of stroke with a high mortality rate. It is also an important cause of permanent disability. Apart from hematoma growth and edema development, inflammatory responses and oxidative stress are responsible for poor outcomes after ICH. Due to its antioxidant, anti-inflammatory and anti-apoptotic properties, melatonin is a neuroprotective molecule against different neurological diseases. The protective roles of melatonin on ICH, particularly in the collagenase-induced ICH model, have not been well studied. The present study aims to explore neuroprotective effects of melatonin against ICH. At 24 hours after ICH induction, rats exhibited neurological deficits with mild loss in body weight (BW). Hematoma was found in the brain parenchyma with ED-1+ activated microglia and TUNEL+ apoptotic cells in the perihematomal region. As an *in vitro* model of ICH, SH-SY5Y cells were treated with red blood cell lysate. This treatment significantly reduced cell viability; however, melatonin (10^{-5} M) restored the cell viability. At 72 hours after ICH, rats treated with melatonin (50 mg/kg) at 2, 24 and 48 hours had reduced perihematomal microglial activation. However, there was no effect on hematoma size or perihematomal apoptosis. We further treated rats with 50 mg/kg melatonin starting at 2 hours and repeating at 24-hour intervals for two or seven more days. Both melatonin treatments improved post-ICH neurological functions, and the effect was most pronounced at 4 days after ICH. Since studies regarding the protective roles of melatonin on ICH remain very limited, our study advances our understanding of the potential use of melatonin as a treatment for ICH.

Key words: Melatonin, stroke, intracerebral hemorrhage, collagenase, neuroprotection, microglia, neurological function, apoptosis

1. INTRODUCTION

Stroke is one of the leading causes of death worldwide. It is responsible for approximate 5.5 million deaths every year (1). Hemorrhagic stroke, which consists of subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ICH), is a severe form of stroke and has a particularly high mortality rate (2). Previous study reported that the five-year survival rate after hemorrhagic stroke was only 26.7% (3). ICH accounts for 10–15% of all strokes and is characterized by bleeding inside the brain parenchyma (4, 5). Not only does ICH cause death, it causes severe, permanent disability in surviving patients (6). Hematoma formation is the primary cause of adverse outcomes in ICH (7). Hematoma expansion from secondary bleeding leads to a further increase in intracranial pressure (8). Brain edema formation after ICH further promotes neurological deficits (9). In addition, red blood cell (RBC) lysis takes place after ICH (10), leading to release of harmful substances, such as iron and hemoglobin, and generation of oxidative stress, which in turns, worsen the prognosis of ICH (11–14).

Melatonin is likely produced in the mitochondria of all cells (15, 16). Its original and primary function is likely an antioxidant as based on its early evolution (17). In mammals, melatonin could activate two high affinity receptors, MT1 (Mel1a, MTNR1A) and MT2 (Mel1b, MTNR1B) (18). Apart from regulating circadian rhythm, MT1 and MT2 receptors play important roles in different physiological functions, including reproduction, cardiovascular regulation and immune function (19–22). Attributing to its direct antioxidant, anti-inflammatory, and anti-apoptotic properties, melatonin is a neuroprotective molecule against different neurological diseases (23–25). Recent studies have also uncovered its functions on neurogenesis promotion (26).

Although there are abundant evidences showing that melatonin is a neuroprotective agent against ischemic stroke (27–29), studies focusing on its effects on hemorrhagic stroke, especially ICH, remain very limited. Also, experimental findings vary from study to study. Two methods are commonly used to induce ICH in the animals, which are by autologous blood injection and collagenase injection (30). In the autologous blood-induced model, high-dose melatonin such as 100 and 150 mg/kg BW was found to promote neuronal survival and reduce apoptosis in the perihematomal region in rats at 24 hours after ICH (31). Also, the high-dose melatonin attenuated post-ICH neurological deficits, brain edema and blood-brain barrier (BBB) leakage (31). In another study, when rats were treated with multiple low-dose melatonin (e.g. 5 mg/kg) after ICH, oxidative stress, DNA damage and neuronal death were reduced (32). However, contradictory results were reported by Li *et al.* (33). It was found that 10 mg/kg melatonin given to the rats at 1 and 4 hours after ICH did not provide any neuroprotection

Collagenase-induced animal model of ICH involves injection of bacterial collagenase into the brain parenchyma, resulting in blood vessel breakdown and bleeding in the surrounding brain tissues (34). In contrast to the autologous blood-induced model, protective roles of melatonin in this model have not been well studied. Till now, only limited number of related articles could be identified (35–37) (Table 1). In addition, a large portion of them reported that melatonin did not exert any protective effect. For instances, it was found that neither multiple doses of 15 mg/kg melatonin nor a single dose of 150 mg/kg melatonin could prevent brain edema formation or neurological deficits after ICH (35, 36). Moreover, the acute effects of melatonin in this ICH model remain unclear (i.e. within 7 days after ICH). Lekic *et al.* (37) have reported that a single dose of 5 and 15 mg/kg melatonin failed to reduce brain water content or improve neurological function in rats at 24 hours after ICH. In the same study, only when multiple doses of 15 mg/kg melatonin were administered, an improvement in forelimb function of the rats was found on the second day

after ICH. Interestingly, however, the protective effect diminished on the third day (37). Since the roles of melatonin in the collagenase-induced model, especially its acute effects, are poorly understood till now, we decided to carry out the present study. In reference to the findings of the previous study (37), we increased the dose and length of treatment of melatonin and would like to see whether the protective effects of melatonin in the collagenase-induced model could be enhanced.

Table 1. Studies of the protective effects of melatonin in the collagenase-induced ICH model.

Type of animal model of ICH	Treatment timeline and dosage	Brain Region	Main findings	References
Type VII collagenase-induced ICH (SD rats)	15 mg/kg melatonin, intraperitoneally injected at 1, 24, 48, and 72 hours after ICH	Ipsilateral hemisphere	Melatonin did not significantly reduce size of infarct area Melatonin did not improve neurological deficits, including cued and spatial learning, spatial memory and sensorimotor coordination in the 8-week test period	Hartman <i>et al.</i> , 2008
Type VII collagenase-induced ICH (SD rats)	15 or 150 mg/kg melatonin, intraperitoneally injected at 15 minutes (the low dose) or 3 hours (both low and high dose) after ICH induction	Ipsilateral cortex	Both 15 and 150 mg/kg melatonin did not exert any protective effect against brain edema formation and neurological deficits 15 mg/kg melatonin (injected at 15 minutes after ICH) reduced lipid peroxidation at 24 hours after ICH	Rojas <i>et al.</i> , 2008
Type VII collagenase-induced ICH (SD rats)	5 or 15 mg/kg melatonin, intraperitoneally injected at 15 minutes or 3 hours after ICH induction (sacrificed at 24 hours after ICH; short-term experiment) 5 or 15 mg/kg melatonin, intraperitoneally injected at 1, 24, 48, and 72 hours after ICH induction (sacrificed at 10 weeks after ICH; long-term experiment)	Ipsilateral cortex	15-minute or 3-hour delayed melatonin treatment (both 5 and 15 mg/kg) did not show any protective effect on brain edema and neurological deficits on 1 day after ICH Repeated 15 mg/kg melatonin treatment (given at 1, 24, 48, and 72 hours after ICH) improved forelimb placing function on second day after ICH but the effect diminished on the third day Repeated 5 mg/kg melatonin treatment failed to provide acute protection but improved learning and memory at 8 weeks after ICH and improved cerebral histopathology at 10 weeks after ICH 3-hour delayed melatonin treatment (both 5 and 15 mg/kg) reduced lipid peroxidation at 24 hours after ICH	Lekic <i>et al.</i> , 2010

2. MATERIALS AND METHODS

2.1. Animals.

Adult male Sprague–Dawley (SD) rats (240–310 g) were housed under controlled temperature with a 12-hour light/dark cycle. They were free to access to food and water. The experimental protocol was approved by the animal ethics committee of the University of Hong Kong (Committee on the Use of Live Animals in Teaching and Research [CULATR 3300-14]).

2.2. Rat model of collagenase-induced ICH.

Surgical procedures were performed according to published studies (38–40). Briefly, anesthetized rat was first held in place on a stereotaxic frame, and a burr hole was drilled over the right hemisphere. 0.2-unit Type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA) in 1.0 μ L saline was then injected slowly into the right striatum over 10 minutes using a 30-gauge (G) needle (at the coordinates: 0.2 mm anterior to the bregma, 3 mm lateral to the midline and 5.5 mm beneath dura). To avoid back-leakage, the injection needle was kept in place for 10 minutes after injection. The burr hole was sealed with bone wax, and the incision was sutured. The sham group received the same surgical procedures, but saline instead of Type IV collagenase was injected.

2.3. *In vivo* experimental design and melatonin treatment.

Three sets of *in vivo* experiments were performed in the present study. First of all, to show that we have successfully established the collagenase-induced model, we examined the changes in the rats at 24 hours after ICH induction (Figure 1A; $n = 4$) and compared with the findings after sham operation (sham group; $n = 4$). The studied parameters were hematoma formation, BW, neurobehavioral performance, apoptosis and microglial activation.

In the second set of experiment, we studied the neuroprotective effects of melatonin at 72 hours after ICH (Figure 3A). After ICH induction, rats were intraperitoneally (i.p.) injected with the vehicle ($n = 5$) or different concentrations of melatonin (at 20 [$n = 3$] or 50 [$n = 5$] mg/kg) at 2, 24 and 48 hours after ICH. Rats were sacrificed at 72 hours, and brain samples were collected for further analysis. Melatonin (Sigma-Aldrich) was diluted to the target concentration using saline with 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Vehicle is saline only containing 5% DMSO. We compared the differences in the following parameters among groups, including daily BW as well as hematoma size, apoptosis and microglial activation at 72 hours.

In the third set of experiment, we studied whether melatonin promoted post-ICH neurobehavioral recovery (Figure 5A). After ICH induction, rats were divided into three groups, which are the vehicle group ($n = 5$), 3-dose melatonin group ($n = 5$) and 8-dose melatonin group ($n = 5$). Vehicle group was given vehicle i.p. at 2 hours and from 1 to 7 days after ICH at 24-hour interval. For the 3-dose melatonin group, rats received 50 mg/kg melatonin i.p. at 2 hours and daily on days 1 and 2 after ICH at 24-hour interval, and then vehicle i.p. was given from 3 to 7 days after ICH at 24-hour interval. For the 8-dose melatonin group, rats received 50 mg/kg melatonin at 2 hours and daily from 1 to 7 days after ICH at 24-hour interval. All the rats were assessed for their neurobehavioral outcomes on days 1, 4 and 7 after ICH. They were all sacrificed on the next day after the last day of the neurobehavioral assessment (i.e., 8 days after ICH). We compared the differences in the following parameters among groups, including hematoma size on day 8 as well as BW and neurobehavioral performance on days 0, 1, 4 and 7.

2.4. Hematoma size measurement.

The rat brain was sliced into 2 mm-thick coronal sections. Area of hematoma on both side of each coronal section was measured using Image J software (NIH, Bethesda, MD, USA) in an

observer-blinded manner. We calculated the sum of hematoma area of all coronal sections in each brain to generate the hematoma size in each animal (in mm³) for comparison among different treatment groups.

2.5. Neurobehavioral assessment.

Rats were assessed for their neurobehavioral performance using the neurological deficit scores (NDS) system before and after ICH induction. The procedures of assessing animals' neurobehavior using NDS system have been described in the previous study (41) and used in other published studies (38–40). Briefly, rats were scored based on their performance in different neurobehavioral tests, including spontaneous-activity test, floor-walking test, tail-raising test, limb-placing tasks and beam-balance test (Supplementary materials). Rats which suffer from more severe deficits will get higher NDS under this scoring system. All assessments were performed by an observer who was blinded to the group identity.

2.6. Immunohistochemistry and stereological analyses.

After sacrificing, rats were perfused transcardially with ice-cold saline and then 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brain samples were post-fixed in 4% PFA overnight at 4°C. After post-fixation, they were immersed in cryoprotectant solution (30% sucrose solution in 0.1 M PB) and stored at 4°C until use. Brain region between 1 mm anterior to the bregma and 3 mm posterior to the bregma as the major site of injury after ICH induction was selected for preparation of 30 µm-thick sections using a cryostat. The brain sections were affixed on gelatine-coated slides and allowed to air-dry overnight. After air-drying, they were kept at -20°C until use.

For ED-1 detection, brain sections were incubated in 0.01 M citric acid (pH 6.0) at 90°C for 10 minutes for antigen retrieval. They were then blocked by 10% normal goat serum and incubated with primary antibody (mouse anti-ED-1, 1:200 dilution, AbD Serotec, Raleigh, NC, USA) overnight at room temperature. After primary-antibody incubation, brain sections were washed with 0.01 M phosphate-buffered saline (PBS) for three times (5 minutes each). After washing with PBS, brain sections were incubated with secondary antibody (biotinylated goat anti-mouse antibody, 1:200 dilution, Vector Laboratories, Burlingame, CA, USA) for 2 hours at room temperature. Then the brain sections were washed with 0.01 M PBS for three times (5 minutes each). To amplify the signal, the brain sections were incubated in avidin-biotin complex solution (1:200, Vector Laboratories) for 1 hour at room temperature. ED-1 signal was finally visualized using the liquid diaminobenzidine (DAB) substrate kit (Invitrogen, Waltham, MA, USA). Afterwards, the brain sections were counterstained with eosin.

To detect TUNEL+ cells, in situ cell death detection kit, POD (Roche, Branchburg, NJ, USA) was used. Brain sections were first incubated in 0.01 M citric acid (pH 6.0) at 90°C for 10 minutes for antigen retrieval. After antigen retrieval, they were incubated in blocking solution for 10 minutes and then permeabilization solution for 2 minutes according to the manufacturer's protocol. Afterwards, TUNEL reaction mixture was prepared using label solution and enzyme solution provided by the manufacturer. Brain sections were further incubated in the TUNEL reaction

mixture for 60 minutes and then converter-POD for 30 minutes at 37°C in a humidified chamber in dark. Finally, TUNEL signals were visualized using the liquid DAB substrate kit (Invitrogen).

For the quantification of ED-1+ and TUNEL+ cells, StereoInvestigator (MicroBrightField, Williston, VT, USA) was utilized. Detailed procedures have been previously described (40). Briefly, the contour of the ipsilateral striatum excluding the hematoma was outlined using the StereoInvestigator software. Systemic random sampling was then performed within the outlined region at 400× magnification. By counting the number of cells in the counting boxes assigned by the program, total number of cells in the region of interest was automatically calculated. In our experiment, at least 3 brain sections were analyzed for each animal. The whole counting process was conducted in a blinded manner. The counting results were presented as the total number of cells per brain section.

2.7. *In vitro* model of ICH and melatonin treatment.

Human SH-SY5Y cells (American Type Culture Collection, Manassas, VA, USA) were used in our *in vitro* experiments. The cells were grown in Dulbecco's Modified Eagle medium/ F12 (DMEM/F12; Invitrogen) with 10 % fetal bovine serum (Invitrogen) in 75 cm² flask. They were maintained in a humidified incubator with 5 % CO₂ at 37°C. To mimic the *in vitro* ICH, SH-SY5Y cells were incubated with RBC lysate. Procedures were performed in reference to the previously described protocol (42, 43). Briefly, we obtained RBCs from human blood by centrifugation. The RBCs collected were then lysed in distilled water at a density of 2 x 10⁹ cells/ml and kept in -80°C until use. To examine the neurotoxic effects of RBC lysate, SH-SY5Y cell viability was determined after incubation with different concentrations of RBC lysate for 24 hours (Figure 2A; n = 3 per group). The present study also examined the potential toxic effects of melatonin on SH-SY5Y cells. Cell viability was determined after treatment with different concentrations of melatonin, ranging from 10⁻³ to 10⁻⁷ M for 24 hours (n = 3 per group). To study neuroprotective effect of melatonin against RBC lysate, SH-SY5Y cells were incubated with 62.5 x 10⁵ cells/ml RBC lysate and different concentrations of melatonin (ranging from 10⁻⁴ to 10⁻⁷ M; n = 3 per group) for 24 hours. Then cell viability was measured.

2.8. Cell viability assay.

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Invitrogen). Cells in 96-well plate were incubated with 0.5 mg/ml MTT for 4 hours. After the incubation, water-insoluble formazan dye formed, and the dye was solubilized using a lysis buffer (10% sodium dodecyl sulfate (SDS; Sigma-Aldrich) with 0.03% hydrochloric acid in distilled water). Absorbance was measured by a 96-well plate spectrophotometer at 540 nm (corrected by 650 nm).

2.9. Statistical analysis.

Data are presented as mean ± SEM in the present study. Statistical difference between two groups was analyzed using Student's t-test. For multiple comparisons, one-way ANOVA followed

by Turkey's test or Dunnett's multiple comparison test was performed. $P < 0.05$ was considered as statistically significant in each test.

3. RESULTS

3.1. Histological and biochemical alterations in brain of rats at 24 hours after ICH induction.

At 24 hours after ICH induction, the hematoma was found in the brain parenchyma of the rats, and it was mainly located in the striatum (Figure 1B). When compared to the sham group, there was a slight but significant decrease in BW ($P < 0.05$; Figure 1C), and high NDS indicated severe neurological deficits ($P < 0.001$; Figure 1D). Moreover, ED 1+ activated microglia and TUNEL+ apoptotic cells increased dramatically in the perihematomal region ($P < 0.01$ and $P < 0.05$, respectively; Figure 1E and 1F).

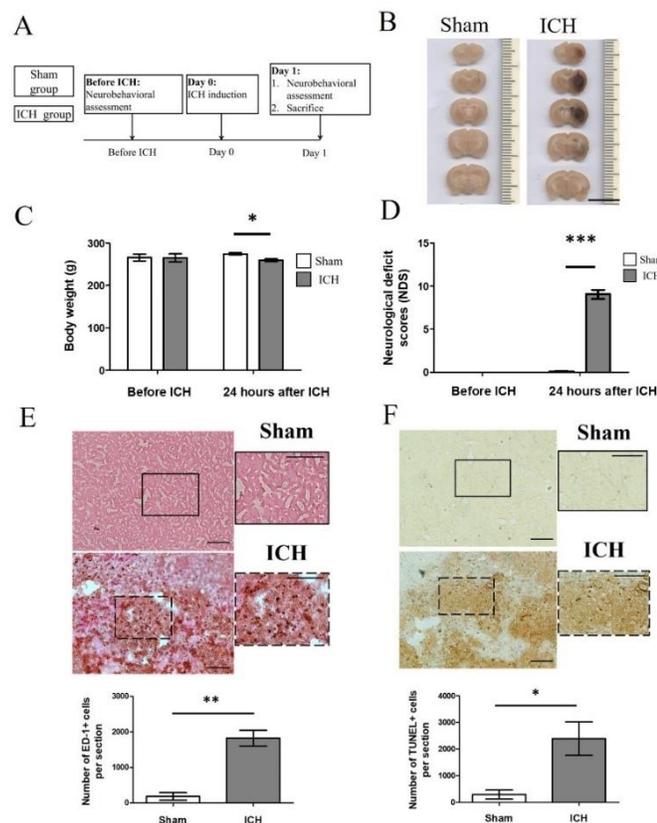


Fig. 1. Histological and biochemical alterations in brain of rats at 24 hours after ICH induction.

(A) Experimental timeline. (B) Images showing coronal brain sections of a sham rat and an ICH rat (2 mm thick; scale bar: 10 mm). (C) BW and (D) NDS of sham and ICH rats. Representative images and quantification of ED-1+ cells (E) and TUNEL+ cells (F) in the striatum/perihematomal region in sham and ICH rats (Scale bar: 100 μ m). Results are expressed as mean \pm SEM; $n = 4$ per group. Data were analyzed by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to sham group.

3.2. Effects of RBC lysate and melatonin on the viability of SH-SY5Y cells.

When SH-SY-5Y cells were treated with different concentrations of RBC lysate, ranging from 31.25×10^5 to 250×10^5 cells/ml for 24 hours as an in vitro ICH model, MTT assay indicated a significant decrease in cell viability in a dose-dependent manner ($P < 0.001$; Figure 2B). SH-SY-5Y cells were incubated with melatonin, ranging from 10^{-3} to 10^{-7} M for 24 hours, to reveal any toxic effect. MTT assay showed that 10^{-3} M melatonin significantly decreased the cell viability ($P < 0.001$; Figure 2C), but other concentrations (10^{-4} to 10^{-7} M) did not cause any difference when compared to control (Figure 2C). SH-SY-5Y cells were treated with 62.5×10^5 cells/ml RBC lysate plus different concentrations of melatonin for 24 hours. We found that 10^{-5} M melatonin significantly ameliorated the decrease in cell viability caused by RBC lysate ($P < 0.05$; Figure 2D).

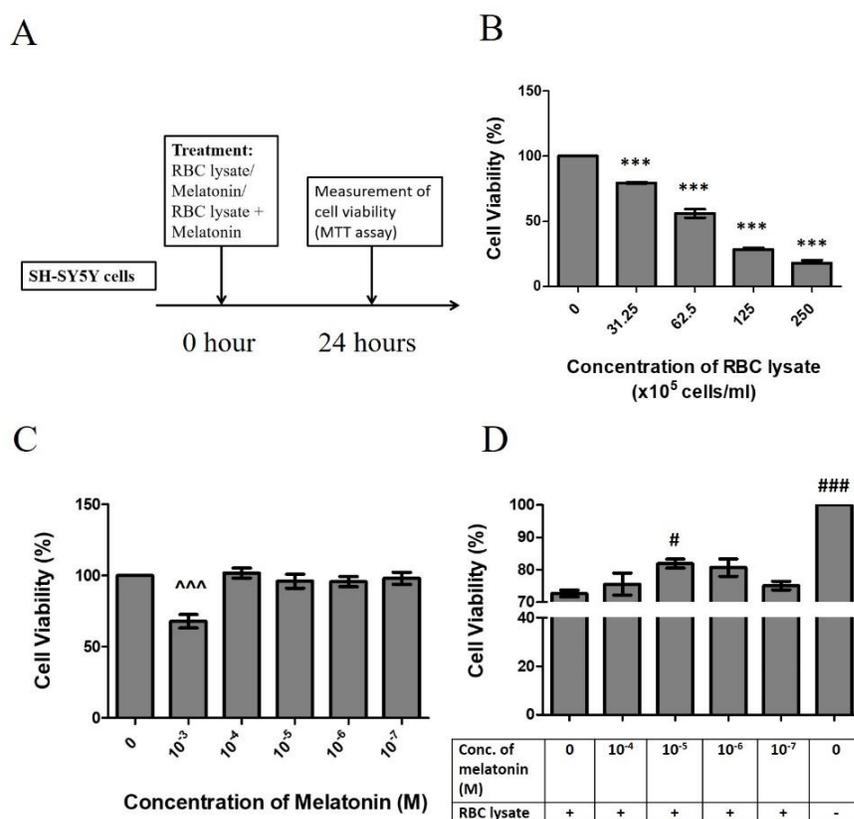


Fig. 2. Effects of RBC lysate and melatonin on the viability of SH-SY5Y cells.

(A) Experimental timeline. (B) RBC lysate decreased viability of SH-SY-5Y cells in a dose-dependent manner. (C) Adverse effect of 10^{-3} M melatonin but not at other concentrations on viability SH-SY-5Y cells. (D) 10^{-5} M melatonin partially reversed the decrease in viability of SH-SY-5Y cells caused by RBC lysate. Cell viability was measured by MTT assay from three independent experiments. Results are expressed as mean \pm SEM; $n = 3$ per group. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test as post-hoc test. *** $P < 0.001$ compared to no RBC lysate treatment (Figure B), ^^ $P < 0.001$ compared to no melatonin treatment (Figure C), and # $P < 0.05$ compared to RBC lysate treatment alone without melatonin and ### $P < 0.001$ compared to RBC lysate treatment (Figure D).

3.3. Effects of melatonin on microglial activation, hematoma size and apoptosis at 72 hours after ICH.

When rats were treated with vehicle or melatonin (20 or 50 mg/kg) at 2, 24 and 48 hours after ICH before sacrifice at 72 hours, there was no significant difference in BW among groups (Figure 3B). In addition, both 20 and 50 mg/kg melatonin did not significantly affect the size of hematoma at 72 hours after ICH (Figure 3C and D). However, 50 mg/kg melatonin significantly decreased the number of ED-1+ activated microglia in the perihematoma region ($P < 0.05$; Figure 4A). There was no significant difference among groups in the number of TUNEL+ apoptotic cells in the perihematoma region (Figure 4B).

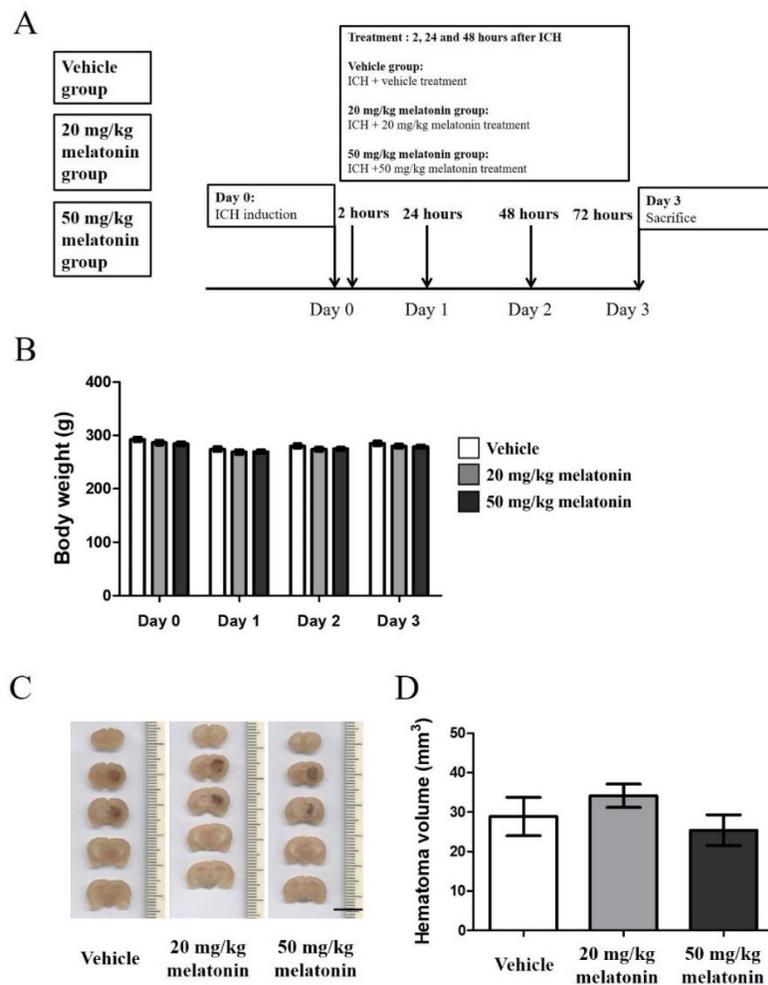


Fig. 3. Effects of melatonin on size of hematoma and BW at 72 hours after ICH.

(A) Experimental timeline. (B) BW. (C) Images showing coronal brain sections of different groups at 72 hours after ICH (2 mm thick; scale bar: 10 mm). (D) Measurement of hematoma size (in mm³). Results are expressed as mean \pm SEM. Vehicle group ($n = 5$), 20 mg/kg melatonin group ($n = 3$), 50 mg/kg melatonin group ($n = 5$). Data were analyzed by one-way ANOVA, followed by Turkey's test as post-hoc test.

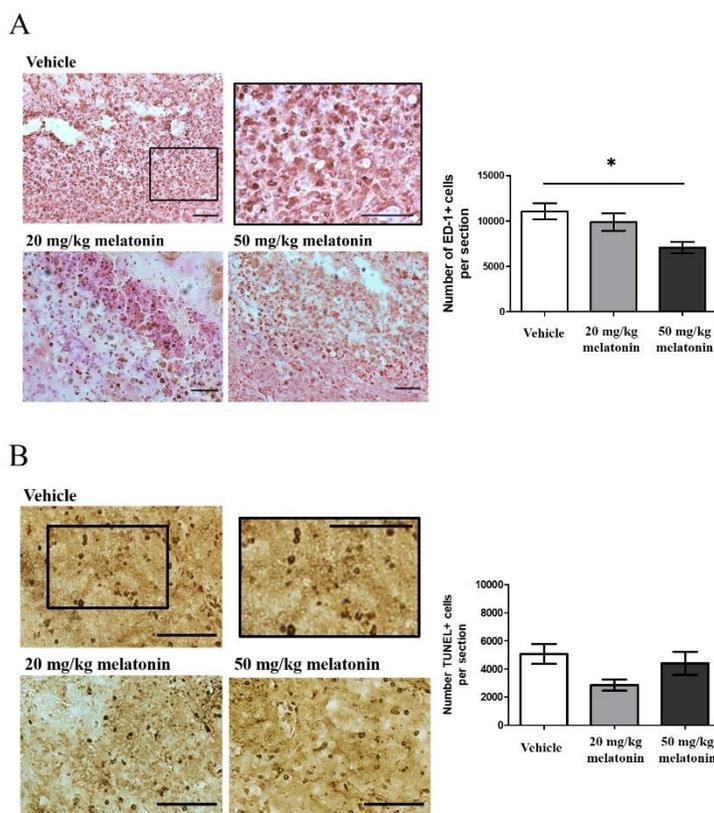


Fig. 4. Effects of melatonin on microglial activation and apoptosis in the perihematomal region at 72 hours after ICH.

(A) Representative images and quantified data showing 50 but not 20 mg/kg melatonin reduced number of ED-1+ activated microglia in the perihematomal region at 72 hours after ICH. (B) Representative images and quantified data showing both 20 and 50 mg/kg melatonin did not reduce number of TUNEL+ apoptotic cells in the perihematomal region at 72 hours after ICH. Results are expressed as mean \pm SEM. Vehicle group ($n = 5$), 20 mg/kg melatonin group ($n = 3$), 50 mg/kg melatonin group ($n = 5$). Data were analyzed by one-way ANOVA, followed by Turkey's test as post-hoc test. * $P < 0.05$ compared to the vehicle group.

3.4. Effects of melatonin on neurological functions after ICH.

When rats were treated with vehicle or melatonin (50 mg/kg) at 2, 24 and 48 hours (i.e. 3-dose groups) after ICH or at 2 hours and repeated at 24-hour interval for 7 doses (i.e. 8-dose groups) before sacrifice on day 8, there was no significant difference among groups in hematoma size on day 8 (Figure 5B and 5C) and BW on days 1, 4 and 7 (Figure 5D). Although there was no significant difference in NDS among groups on day 1 after ICH (Figure 5E), both 3-dose and 8-dose melatonin treatments significantly improved neurological functions on day 4 after ICH when compared to the vehicle groups ($P < 0.05$; Figure 5E). On day 7 after ICH, both melatonin-treated groups still performed better when compared to the vehicle group, but the differences were not statistically significant (Figure 5E).

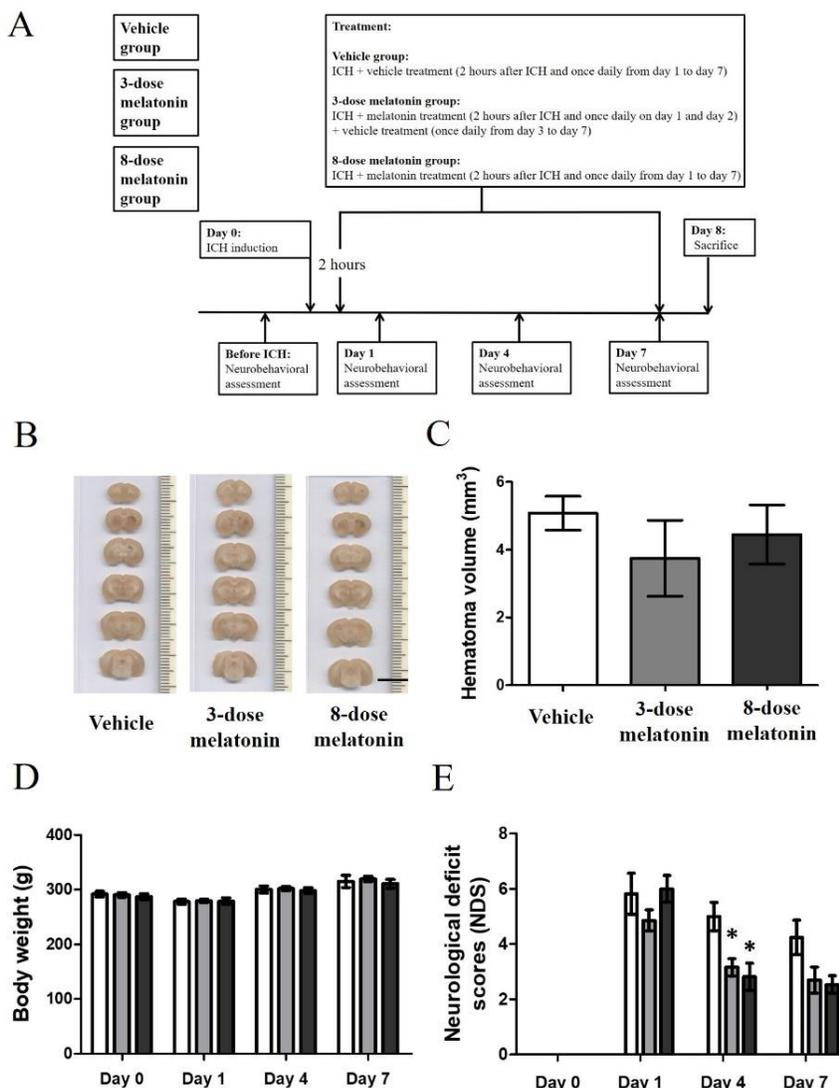


Fig. 5. Effects of melatonin on neurological functions after ICH.

(A) Experimental timeline. (B) Images showing coronal brain sections of different groups on day 8 after ICH (2 mm thick; scale bar: 10 mm). (C) Measurement of size of hematoma. (D) BW. (E) Melatonin improved neurological functions after ICH. Results are expressed as mean \pm SEM; $n = 5$ in each group. Data were analyzed by one-way ANOVA, followed by Turkey's test as post-hoc test. * $P < 0.05$ compared to the vehicle group.

4. DISCUSSIONS

ICH is a severe form of stroke. It causes permanent disability in patients and has a high mortality rate (2, 3). Apart from hematoma growth and edema development, inflammatory responses and oxidative stress are responsible for poor outcomes after ICH (44, 45). As a free radical scavenger, melatonin is well-known for its antioxidant and anti-inflammatory properties (46). Therefore, it is an ideal candidate to be evaluated as a treatment for ICH. However, its

protective roles on hemorrhagic stroke, especially on ICH are poorly understood. The present study has investigated the protective roles of melatonin on ICH using the collagenase-induced model.

We started our study by assessing the changes in rats at 24 hours after ICH induction. Comparing with sham rats, ICH rats had slightly reduced BW, and hematoma was found in the striatum. The ICH rats also showed severe neurological deficits. Moreover, there were dramatic increases in numbers of ED-1+ activated microglia and TUNEL+ apoptotic cells in the perihematomal region. We then examined the protective effects of melatonin in an *in vitro* model of ICH. The RBC-lysate model could well-mimic ICH condition *in vitro*, and it has not been previously used to assess neuroprotective effects of melatonin. Previous studies used oxyhemoglobin, hemin and thrombin as *in vitro* models of ICH (32, 47, 48). Using the RBC-lysate model, our results demonstrated that 10^{-5} M melatonin could significantly increase viability of SH-SY5Y cells after RBC lysate treatment.

We then tested the protective effects of melatonin on ICH *in vivo*. Given the results of previous study showing that single dose of 5 and 15 mg/kg melatonin did not provide any neuroprotection at 24 hours after ICH in the collagenase-induced model (37), we decided to increase the dose and period of treatment. The rats were treated with 20 or 50 mg/kg melatonin at 2, 24 and 48 hours after ICH induction and sacrificed them at 72 hours. According to our findings from using higher dose of melatonin than the previous study (37), there was no difference in the hematoma size between the melatonin-treated rats and vehicle-treated rats at 72 hours after ICH. To our best knowledge, the protective effects of melatonin against apoptosis and microglial activation in the collagenase-induced model have not been reported before. In this study, we found that multiple doses of 50 mg/kg melatonin significantly reduced ED-1+ activated microglia in the perihematomal region after ICH, although apoptosis was not ameliorated.

Microglial activation has been found to be beneficial to ICH. Microglia are responsible for removal and cleanup of cellular debris from hematoma (49). However, their activation also promotes production of a wide range of proinflammatory cytokines and chemokines, including TNF- α , IL-1 β , IL-6 and CXCL2 (50, 51), which in turns leads to neuroinflammation and causes secondary injury in ICH. It was reported that microglia reached their peak number in the perihematomal region at 72 hours after ICH (51). Coincidentally, the present study showed that multiple doses of 50 mg/kg melatonin decreased microglia activation at this time point. Given that inhibiting microglial activation using minocycline has been found to be effective in improving ICH outcomes (51), we believed that the dosage of melatonin used in our study is also beneficial to ICH. Therefore, whether melatonin could promote post-ICH neurological functional recovery was investigated.

The rats were treated with 50 mg/kg melatonin starting at 2 hours and repeating daily for two or seven times after ICH. We assessed their neurobehavioral outcomes on days 1, 4 and 7 after ICH. It was found that both 3-dose and 8-dose melatonin groups significantly improved neurological functions of the rats on day 4 after ICH. On day 7 after ICH, both melatonin-treated groups still performed better than the vehicle-treated group, but the differences were not statistically significant.

In summary, our study showed that multiple doses of 50 mg/kg melatonin could inhibit post-ICH microglial activation, which may be responsible for the improvement in neurological functions of the rats after ICH. Based on our current findings, future studies should be focused on

the following aspects. First, since multiple doses of 50 mg/kg melatonin reduced microglial activation but not apoptosis after ICH, protective effects of even higher doses of melatonin or longer duration of treatment on ICH can be evaluated. Second, as molecular mechanisms regarding the protective effects of melatonin in the collagenase-induced model remain poorly understood and melatonin possesses anti-microglial activation property, the effects of melatonin on different microglial-related inflammatory pathways can be investigated in this ICH model. Third, the role of melatonin in enhancing recovery after ICH via promoting endogenous neurogenesis can be explored in the collagenase-induced model because recent studies showed that melatonin protected against different neurological diseases by stimulation of endogenous neurogenesis (26).

Although much evidence indicated that melatonin is neuroprotective against ischemic stroke, studies regarding its role on ICH, especially in the collagenase-induced model, remain very limited. Since collagenase-induced model could well-mimic clinical ICH condition of acute cerebrovascular rupture with blood-brain barrier breakdown (52) and well-demonstrate histological changes in the brain (53), it is a reliable experimental model for the study of ICH. But surprisingly, the protective roles of melatonin in this model are poorly studied. The present study advances our understanding of the roles of melatonin in collagenase-induced model, which will provide insight into the potential use of melatonin as a treatment for ICH.

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AUTHORSHIP

JWHL and RTFC were involved in development of the hypotheses and experimental design. JWHL conducted the majority of the experiments. JWHL and RTFC performed the data analyses. JWHL drafted the manuscript. RTFC critically revised the manuscript. JWHL and RTFC approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

1. GBD 2016 Stroke Collaborators (2019) Global, regional, and national burden of stroke, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* **18**: 439–458.

2. Wu H-J, Wu C, Niu H-J, Wang K, Mo L-J, Shao A-W, Dixon BJ, Zhang J-M, Yang S-X, Wang Y-R (2017) Neuroprotective mechanisms of melatonin in hemorrhagic stroke. *Cell Mol. Neurobiol.* **37**: 1173–1185.
3. Kojic B, Burina A, Hodzic R, Pasic Z, Sinanovic O (2009) Risk factors impact on the long-term survival after hemorrhagic stroke. *Med. Arch.* **63**: 203–206.
4. Keep RF, Hua Y, Xi G (2012) Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. *Lancet Neurol.* **11**: 720–731.
5. Qureshi AI, Tuhim S, Broderick JP, Batjer HH, Hondo H, Hanley DF (2001) Spontaneous intracerebral hemorrhage. *N. Engl. J. Med.* **344**: 1450–1460.
6. Øie LR, Madsbu MA, Solheim O, Jakola AS, Giannadakis C, Vorhaug A, Padayachy L, Jensberg H, Dodick D, Salvesen Ø, Gulati S (2018) Functional outcome and survival following spontaneous intracerebral hemorrhage: A retrospective population-based study. *Brain Behav.* **8**: e01113.
7. Brott T, Kothari JR, Barsan W, Tomsick T, Sauerbeck L, Spilker J, Duldner J, Khoury J (1997) Early hemorrhage growth in patients with intracerebral hemorrhage. *Stroke* **28**: 1–5.
8. Mayer SA, Rincon F (2005) Treatment of intracerebral haemorrhage. *Lancet Neurol.* **4**: 662–672.
9. Mayer SA, Sacco RL, Shi T, Mohr JP (1994) Neurologic deterioration in noncomatose patients with supratentorial intracerebral hemorrhage. *Neurology* **44**: 1379–1384.
10. Aronowski J, Zhao X (2011) Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. *Stroke* **42**: 1781–1786.
11. Righy C, Bozza MT, Oliveira MF, Bozza FA (2016) Molecular, cellular and clinical aspects of intracerebral hemorrhage: are the enemies within? *Curr. Neuropharmacol.* **14**: 392–402.
12. Huang F-P, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT (2002) Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products. *J. Neurosurg.* **96**: 287–293.
13. Xi G, Keep RF, Hoff JT (1998) Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J. Neurosurg.* **89**: 991–996.
14. Hu X, Tao C, Gan Q, Zheng J, Li H, You C (2016) Oxidative stress in intracerebral hemorrhage: sources, mechanisms, and therapeutic targets. *Oxid. Med. Cell Longev.* **2016**: 3215391.
15. Tan DX, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ (2013) Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin's primary function and evolution in eukaryotes. *J. Pineal Res.* **54**: 127–138.
16. Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, Leronni D, Mihalik AC, He Y, Cecon E, Wehbi VL, Kim J, Heath BE, Baranova OV, Wang X, Gable MJ, Kretz ES, Di Benedetto G, Lezon TR, Ferrando LM, Larkin TM, Sullivan M, Yablonska S, Wang J, Minnigh MB, Guillaumet G, Suzenet F, Richardson RM, Poloyac SM, Stolz DB, Jockers R, Witt-Enderby PA, Carlisle DL, Vilaradaga JP, Friedlander RM (2017) Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proc. Natl. Acad. Sci. U.S.A.* **114**: E7997–E8006.

17. Manchester LC, Poeggeler B, Alvares FL, Ogden GB, Reiter RJ (1995) Melatonin immunoreactivity in the photosynthetic prokaryote *Rhodospirillum rubrum*: implications for an ancient antioxidant system. *Cell Mol. Biol. Res.* **41**: 391–395.
18. Leung JW, Lau WK, Lau BW, Yee BK (2019) A commentary on the therapeutic potential of melatonin and its analogues in CNS conditions. *Psychiatry and Neuroscience Update : From Translational Research to a Humanistic Approach - Volume III*, eds Gargiulo PÁ, Mesones Arroyo HL (Springer International Publishing), pp. 177–186.
19. Stein RM, Kang HJ, McCorvy JD, Glatfelter GC, Jones AJ, Che T, Slocum S, Huang XP, Savych O, Moroz YS, Stauch B, Johansson LC, Cherezov V, Kenakin T, Irwin JJ, Shoichet BK, Roth BL, Dubocovich ML (2000) Virtual discovery of melatonin receptor ligands to modulate circadian rhythms. *Nature* **579**: 609–614.
20. González-Arto M, Aguilar D, Gaspar-Torruibia E, Gallego M, Carvajal-Serna M, Herrera-Marcos LV, Serrano-Blesa E, Hamilton TR, Pérez-Pé R, Muiño-Blanco T, Cebrián-Pérez JA, Casao A (2017) Melatonin MT₁ and MT₂ receptors in the ram reproductive tract. *Int. J. Mol. Sci.* **18**: 662.
21. Masana MI, Doolen S, Ersahin C, Al-Ghoul WM, Duckles SP, Dubocovich ML, Krause DN (2002) MT(2) melatonin receptors are present and functional in rat caudal artery. *J. Pharmacol. Exp. Ther.* **302**: 1295–1302.
22. Carrillo-Vico A, Lardone PJ, Alvarez-Sánchez N, Rodríguez-Rodríguez A, Guerrero JM (2013) Melatonin: buffering the immune system. *Int. J. Mol. Sci.* **14**: 8638–8683.
23. Alghamdi BS (2018) The neuroprotective role of melatonin in neurological disorders. *J. Neurosci. Res.* **96**: 1136–1149.
24. Monteiro MC, Coleman MD, Hill EJ, Prediger RD, Maia CSF (2017) Neuroprotection in neurodegenerative disease: from basic science to clinical applications. *Oxid. Med. Cell Longev.* **2017**: 2949102.
25. Vincent B (2018) Protective roles of melatonin against the amyloid-dependent development of Alzheimer’s disease: a critical review. *Pharmacol. Res.* **134**: 223–237.
26. Leung JW, Cheung K, Ngai SP, Tsang HW, Lau BW (2020) Protective effects of melatonin on neurogenesis impairment in neurological disorders and its relevant molecular mechanisms. *Int. J. Mol. Sci.* **21**: 5645.
27. Andrabi SS, Parvez S, Tabassum H (2015) Melatonin and ischemic stroke: mechanistic roles and action. *Adv. Pharmacol. Sci.* **2015**: 384750 (2015).
28. Macleod MR, O’Collins T, Horky LL, Howells DW, Donnan GA (2005) Systematic review and meta-analysis of the efficacy of melatonin in experimental stroke. *J. Pineal Res.* **38**: 35–41.
29. Reiter RJ, Sainz RM, Lopez-Burillo S, Mayo JC, Manchester LC, Tan DX (2003) Melatonin ameliorates neurologic damage and neurophysiologic deficits in experimental models of stroke. *Ann. N. Y. Acad. Sci.* **993**: 35–47; discussion 48–53.
30. Manaenko A, Chen H, Zhang JH, Tang J (2011) Comparison of different preclinical models of intracerebral hemorrhage. *Acta Neurochir. Suppl.* **111**: 9–14.
31. Xu W, Lu X, Zheng J, Li T, Gao L, Lenahan C, Shao A, Zhang J, Yu J (2018) Melatonin protects against neuronal apoptosis via suppression of the ATF6/CHOP pathway in a rat model of intracerebral hemorrhage. *Front. Neurosci.* **12**: 638.

32. Wang Z, Zhou F, Dou Y, Tian X, Liu C, Li H, Shen H, Chen G (2018) Melatonin alleviates intracerebral hemorrhage-induced secondary brain injury in rats via suppressing apoptosis, inflammation, oxidative stress, DNA damage, and mitochondria injury. *Transl. Stroke Res.* **9**: 74–91.
33. Li Z, Liang G, Xue Y, Liu Y (2009) Effects of combination treatment of dexamethasone and melatonin on brain injury in intracerebral hemorrhage model in rats. *Brain Res.* **1264**: 98–103.
34. MacLellan CL, Silasi G, Auriat AM, Colbourne F (2010) Rodent models of intracerebral hemorrhage. *Stroke* **41**: S95–98.
35. Hartman RE, Rojas HA, Lekic T, Ayer R, Lee S, Jadhav V, Titova E, Tang J, Zhang JH (2008) Long-term effects of melatonin after intracerebral hemorrhage in rats. *Acta Neurochir. Suppl.* **105**: 99–100.
36. Rojas H, Lekic T, Chen W, Jadhav V, Titova E, Martin RD, Tang J, Zhang J (2008) The antioxidant effects of melatonin after intracerebral hemorrhage in rats. *Acta Neurochir. Suppl.* **105**: 19–21.
37. Lekic T, Hartman R, Rojas H, Manaenko A, Chen W, Ayer R, Tang J, Zhang JH (2010) Protective effect of melatonin upon neuropathology, striatal function, and memory ability after intracerebral hemorrhage in rats. *J. Neurotrauma* **27**: 627–637.
38. Sang YH, Liang YX, Liu LG, Ellis-Behnke RG, Wu WT, So KF, Cheung RT (2013) Rat model of intracerebral hemorrhage permitting hematoma aspiration plus intralesional injection. *Exp. Anim.* **62**: 63–69.
39. Sang LY, Liang YX, Li Y, Wong WM, Tay DK, So KF, Ellis-Behnke RG, Wu W, Cheung RT. (2015) A self-assembling nanomaterial reduces acute brain injury and enhances functional recovery in a rat model of intracerebral hemorrhage. *Nanomedicine* **11**: 611–620.
40. Sang YH, Su HX, Wu WT, So KF, Cheung RT (2011) Elevated blood pressure aggravates intracerebral hemorrhage-induced brain injury. *J. Neurotrauma* **28**: 2523–2534.
41. Liu L, Cheung RT (2013) Effects of pretreatment with a combination of melatonin and electroacupuncture in a rat model of transient focal cerebral ischemia. *Evid. Based Complement. Alternat. Med.* **2013**: 953162.
42. Yang Z, Liu B, Zhong L, Shen H, Lin C, Lin L, Zhang N, Yuan B (2015) Toll-like receptor-4-mediated autophagy contributes to microglial activation and inflammatory injury in mouse models of intracerebral haemorrhage. *Neuropathol. Appl. Neurobiol.* **41**: e95–e106.
43. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, Aronowski J (2009) Neuroprotective role of haptoglobin after intracerebral hemorrhage. *J. Neurosci.* **29**: 15819–15827.
44. Mracsko E, Veltkamp R (2014) Neuroinflammation after intracerebral hemorrhage. *Front. Cell. Neurosci.* **8**: 388.
45. Duan X, Wen Z, Shen H, Shen M, Chen G (2016) Intracerebral hemorrhage, oxidative stress, and antioxidant therapy. *Oxid. Med. Cell Longev.* **2016**: 1203285.
46. Tan DX, Manchester LC, Esteban-Zubero E, Zhou Z, Reiter RJ (1995) Melatonin as a potent and inducible endogenous antioxidant: synthesis and metabolism. *Molecules* **20**: 18886–18906.
47. Chen X, Xi Z, Liang H, Sun Y, Zhong Z, Wang B, Bian L, Sun Q (2019) Melatonin prevents mice cortical astrocytes from hemin-induced toxicity through activating PKC α /Nrf2/HO-1 signaling in vitro. *Front. Neurosci.* **13**: 760.

48. Tang J, Chen R, Wang L, Yu L, Zuo D, Cui G, Gong X (2020) Melatonin attenuates thrombin-induced inflammation in BV2 cells and then protects HT22 cells from apoptosis. *Inflammation* **43**: 1959–1970.
49. Wang J (2010) Preclinical and clinical research on inflammation after intracerebral hemorrhage. *Prog. Neurobiol.* **92**: 463–477.
50. Wang J, Doré S (2007) Inflammation after intracerebral hemorrhage. *J. Cereb. Blood Flow Metab.* **27**: 894–908.
51. Taylor RA, Sansing LH (2013) Microglial responses after ischemic stroke and intracerebral hemorrhage. *Clin. Dev. Immunol.* **2013**: 746068.
52. Lan X, Han X, Li Q, Yang Q-W, Wang J (2017) Modulators of microglial activation and polarization after intracerebral haemorrhage. *Nat. Rev. Neurol.* **13**: 420–433.
53. Del Bigio MR, Yan HJ, Buist R, Peeling J (1996) Experimental intracerebral hemorrhage in rats. Magnetic resonance imaging and histopathological correlates. *Stroke* **27**: 2312–2319; discussion 2319–2320.



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