Neuroprotection by Melatonin after Germinal Matrix Hemorrhage in Neonatal Rats

Tim Lekic, Anatol Manaenko, William Rolland, Kelly Virbel, Richard Hartman, Jiping Tang, and John H. Zhang

Abstract *Background*: Germinal matrix hemorrhage (GMH) is a devastating neurological disorder of very low birth weight premature infants that leads to post-hemorrhagic hydrocephalus, cerebral palsy, and mental retardation. Melatonin is a potent antioxidant known to reverse free-radical mediated injury in the brain. This study investigated the effect of melatonin treatment after GMH injury.

Methods: Clostridial collagenase was infused into the right germinal matrix region of neonatal rats with stereotaxic technique. Cognitive function, sensorimotor ability, cerebral, cardiac and splenic growths were measured in juvenile animals.

Results: Systemic melatonin treatment ameliorated cognitive and sensorimotor dysfunction at the juvenile developmental stage. This hormone also normalized brain atrophy, splenomegaly, and cardiac hypertrophy consequences at 1 month after injury.

Conclusion: This study supports the role of free radicals in acute neonatal hemorrhagic brain injury. Melatonin is an effective antioxidant that can protect the infant's brain from the post-hemorrhagic consequences of mental retardation and cerebral palsy. Further mechanistic studies are warranted to determine the mechanisms behind these neuroprotective effects.

Keywords Melatonin · Neurological deficits · Stroke · Experimental

Introduction

Germinal matrix hemorrhage (GMH) is a clinical condition of very low birth weight (VLBW ≤ 1,500 g) premature neonates in which immature blood vessels rupture within the anterior caudate (sub-ventricular) brain region during the first 7 days of life [1, 2]. This affects approximately 3.5/1,000 births in the United States each year [3]. The consequences of this brain injury are hydrocephalus (post-hemorrhagic ventricular dilation), developmental delay, and a lifetime of cerebral palsy and mental retardation [4, 5]. Although this is an important disease, experimental studies investigating thearapeutic modalities are lacking [6].

Interventions targeting free-radical mechanisms have been shown to be neuroprotective after brain hemorrhage in adult rats [7–11]. Thrombin is released from the clot, and erythrocytes undergo lysis to release the neurotoxins hemoglobin, heme, and iron [12–14]. These will, in turn, diffusely oxidatively damage proteins, lipid, and DNA within the first day after brain hemorrhage [15–21]. Melatonin is a potent antioxidant and free-radical scavenger [22–24] shown to inhibit free-radical-associated red blood cell lysis [25], hemoglobin degradation [26], neuronal cell death [27], and hippocampal and nigrostriatal degeneration [28].

In light of this evidence, we hypothesized that melatonin can be a reasonable therapeutic modality for the amelioration of hemorrhage-mediated free-radical brain injury mechanisms in neonatal rats. This intervention could improve juvenile cognitive and sensorimotor outcomes after neonatal germinal matrix hemorrhage.

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Methods and Materials

Animal Groups and General Procedures

This study was in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Institutional Animal Care and Use Committee at Loma Linda University. Timed pregnant Sprague-Dawley rats were housed with food and water available *ad libitum*. Treatment consisted of melatonin (Sigma Aldrich) dissolved in 10% ethanol and diluted with 0.9% normal saline. This was administered (I.P.) at 60 min after collagenase infusion using treatment dosages of 5 mg/kg and 10 mg/kg. Postnatal day 7 (P7) pups were blindly assigned to the following ($n=8$/group): sham (naive), needle (control), GMH (collagenase-infusion), GMH + 5 mg/kg melatonin, and GMH + 10 mg/kg melatonin. All groups were evenly divided within each litter.

Experimental Model of GMH

Using an aseptic technique, rat pups were gently anaesthetized with 3% isoflurane (in mixed air and oxygen) while placed prone on a stereotaxic frame. Betadine sterilized the surgical scalp area, which was incised in the longitudinal plane to expose the skull and reveal the bregma. The following stereotactic coordinates were determined: 1 mm (anterior), 1.5 mm (lateral), and 3.5 mm (ventral) from the bregma. A bore hole (1 mm) was drilled, into which a 27-gauge needle was inserted at a rate of 1 mm/min. A microinfusion pump (Harvard Apparatus, Holliston, MA) infused 0.3 units of clostridial collagenase VII-S (Sigma, St Louis, MO) through a Hamilton syringe. The needle remained in place for an additional 10 min after injection to prevent "back-leakage." After needle removal, the burr hole was sealed with bone wax, the incision sutured closed, and the animals allowed to recover. The entire surgery took on average 20 min. Upon recovering from anesthesia, the animals were returned to their dams. Needle controls consisted of needle insertion alone without collagenase infusion, while naïve animals did not receive any surgery.

Cognitive Measures

Higher order brain function was assessed during the third week after collagenase infusion. The T-Maze assessed short-term (working) memory [29]. Rats were placed into the stem (40 cm × 10 cm) of a maze and allowed to explore until one arm (46 cm × 10 cm) was chosen. From the sequence of ten trials, of left and right arm choices, the rate of spontaneous alternation (0% = none and 100% = complete, alternations/trial) was calculated, as routinely performed [30, 31]. The Morris water maze assessed spatial learning and memory on four daily blocks, as described previously in detail [11, 32]. The apparatus consisted of a metal pool (110 cm diameter), filled to within 15 cm of the upper edge, with a platform (11 cm diameter) for the animal to escape onto, which changed location for each block (maximum = 60 s/trial), and data were digitally analyzed by Noldus Ethovision tracking software. Cued trials measured place learning with the escape platform visible above water. Spatial trials measured spatial learning with the platform submerged, and probe trials measured spatial memory once the platform was removed. For the locomotor activity, in an open field, the path length in open-topped plastic boxes (49 cm long, 35.5 cm wide, 44.5 cm tall) was digitally recorded for 30 min and analyzed by Noldus Ethovision tracking software [32].

Sensorimotor Function

At 4 weeks after collagenase infusion, the animals were tested for functional ability. Neurodeficit was quantified using a summation of scores (maximum = 12) given for (1) postural reflex, (2) proprioceptive limb placing, (3) back pressure towards the edge, (4) lateral pressure towards the edge, (5) forelimb placement, and (6) lateral limb placement (2 = severe, 1 = moderate, 0 = none), as routinely performed [30]. For the rotarod, striatal ability was assessed using an apparatus consisting of a horizontal, accelerated (2 rpm/5 s), rotating cylinder (7 cm-diameter × 9.5 cm-wide) requiring continuous walking to avoid falling recorded by the photobeam circuit (Columbus Instruments) [11, 32]. For foot fault, the number of complete limb missteps through the openings was counted over 2 min while exploring over an elevated wire (3 mm) grid (20 cm × 40 cm) floor [31].

Assessment of Treatment upon Cerebral and Somatic Growth

At the completion of experiments, the brains were removed and hemispheres separated by midline incision (loss of brain weight has been used as the primary variable to estimate brain damage in juvenile animals after neonatal brain injury [33]). For organ weights, the spleen and heart were separated from surrounding tissue and vessels. The quantification was performed using an analytical microbalance (model AE 100; Mettler Instrument Co., Columbus, OH) capable of 1.0 μg precision.

Statistical Analysis

Significance was considered at $P<0.05$. Data were analyzed using analysis of variance (ANOVA) with repeated measures (RM-ANOVA) for long-term neurobehavior. Significant interactions were explored with conservative Scheffé *post hoc* and Mann-Whitney rank sum test when appropriate.

Results

Collagenase infusion led to significant cognitive dysfunction in the T-maze (working) memory and water maze (spatial) learning and memory (Figs. 1a–c, $P<0.05$). Melatonin treatment normalized T-maze deficits (Fig. 1a, $P>0.05$ compared to controls) and water maze (spatial) learning deficits (Fig. 1b, $P<0.05$ compared to GMH), without improving spatial memory (Fig. 1c, $P>0.05$). Both doses of melatonin also normalized ($P<0.05$) the significant sensorimotor dysfunction (compared to juvenile GMH animals), demonstrated by the neurodeficit score, number of foot faults, and accelerating rotarod falling latency (Figs. 2a–c, $P<0.05$). Broad cytoprotection by melatonin was confirmed by the improvement upon brain atrophy (Fig. 3a, $P<0.05$ compared to GMH), and normalization of peripheral splenomegaly and cardiomegaly (Fig. 3b and c, $P>0.05$ compared to controls) at 4 weeks after injury.

Discussion

These results indicate that systemic melatonin treatment after neonatal injury can reduce long-term brain atrophy, and return sensorimotor and cognitive function to near-normal levels in juvenile animals. In support of the findings from others, these outcomes provide preliminary evidence about the importance of oxidative stress mechanisms on outcomes after neonatal GMH [7, 8, 10].

Beyond the improvements in sensorimotor function and brain atrophy, the cognitive normalization by melatonin could have mechanistic benefits beyond reductions of periventricular free radical injury. Hippocampal neurons have receptors for melatonin [34, 35], upon which can be modulated excitability, synaptic transmission, and plasticity [35–38]. These targets could augment melatonin's neuroprotective effects beyond a reduction of oxidative stress alone [38–44]. Mechanistic studies can investigate these processes further, as a window of opportunity for lasting neuroprotection after neonatal GMH.

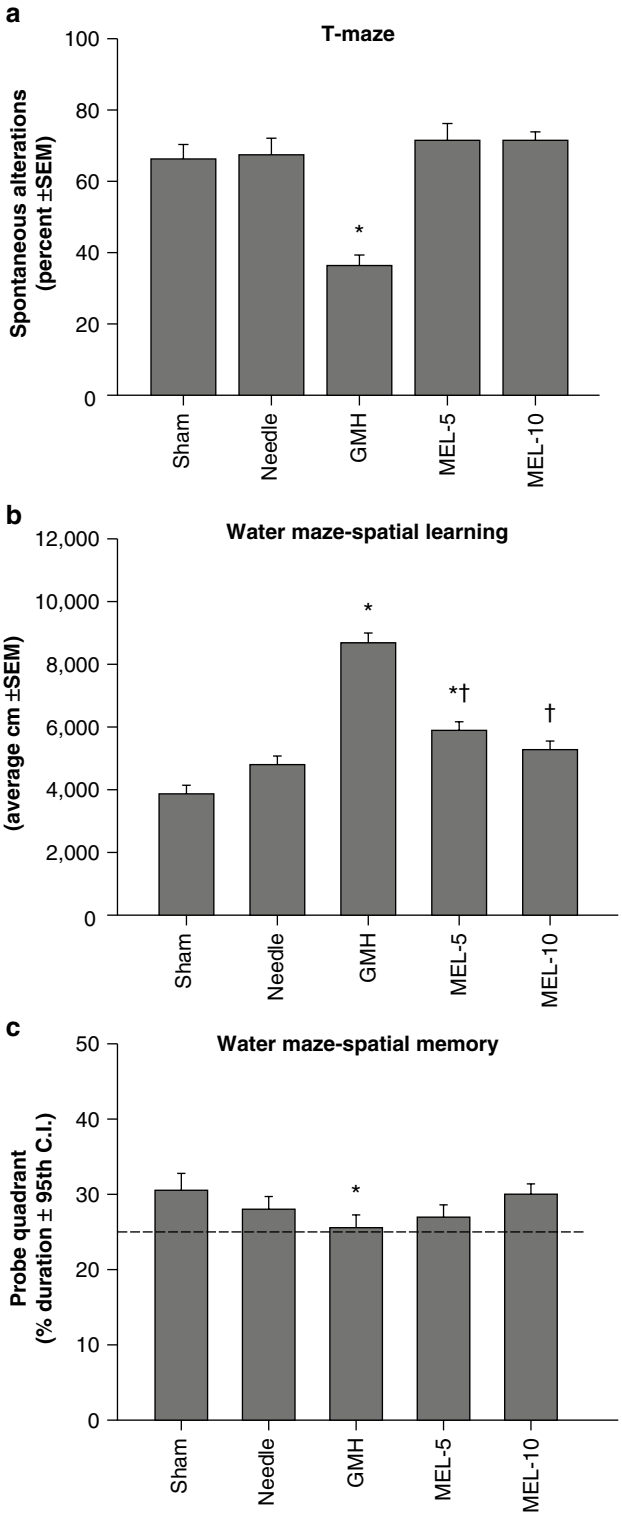

Fig. 1 Cognitive function normalization in juvenile rats by melatonin (MEL) after neonatal GMH. Higher order function was measured at the third week after collagenase infusion: (**a**) T-maze, (**b**) spatial learning water maze, (**c**) spatial memory (Probe) water maze. Values expressed as mean ± 95th CI (probe quadrant) or mean ± SEM (all others), $n=8$ (per group), $^{*}P<0.05$ compared with controls (sham and needle trauma), and $^{\dagger}P<0.05$ compared with GMH

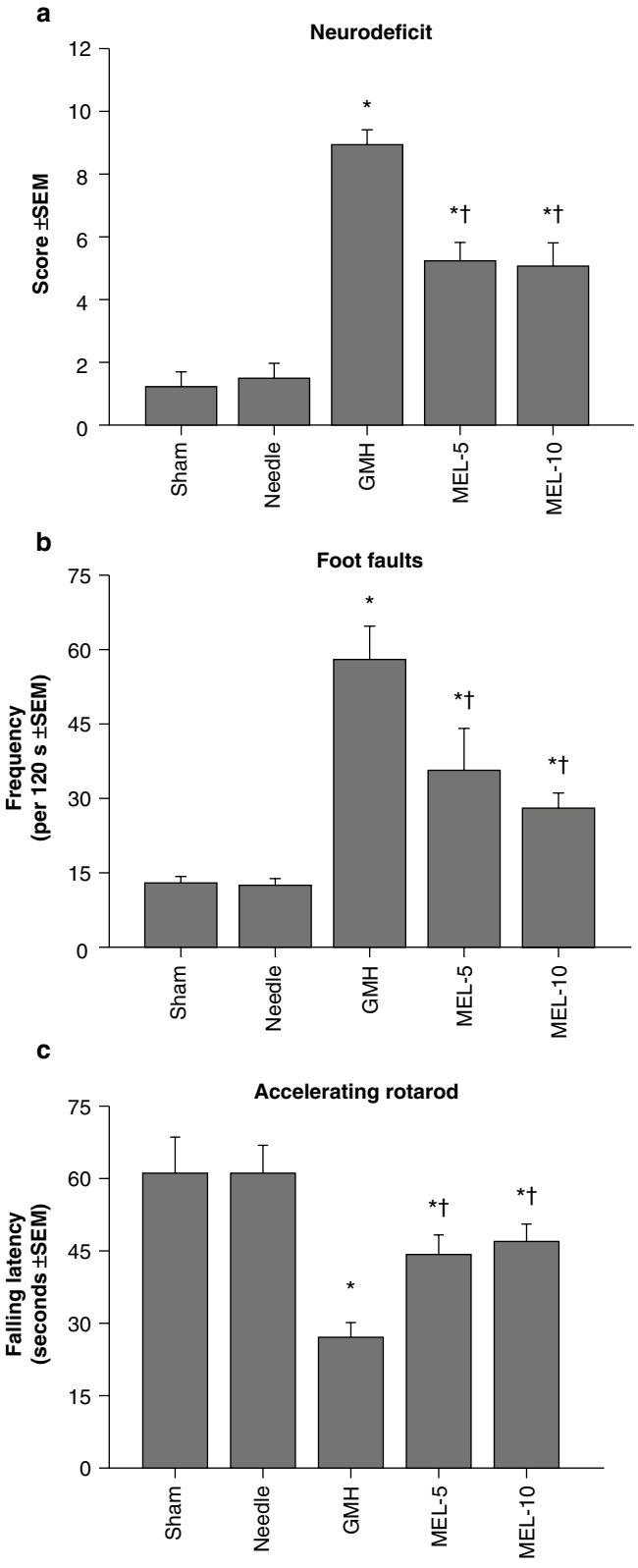

Fig. 2 Sensorimotor function normalization in juvenile rats by melatonin (MEL) after neonatal GMH. Cerebral palsy measurements were performed in the juveniles at 1 month after collagenase infusion: (**a**) neurodeficit score, (**b**) foot faults and (**c**) rotarod. Values expressed as mean ± SEM, $n = 8$ (per group), $^*P < 0.05$ compared with controls (sham and needle trauma), and $\dagger P < 0.05$ compared with GMH

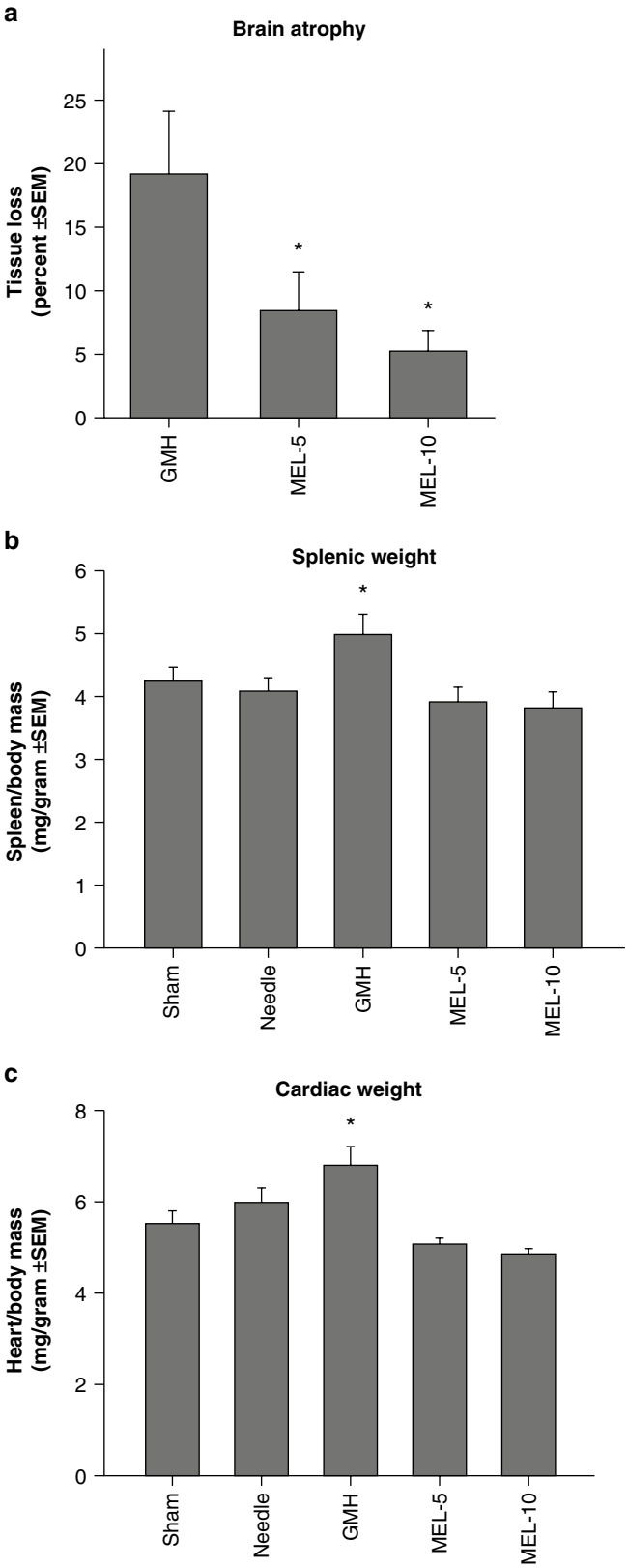

Fig. 3 Cerebral and somatic growth normalization in juvenile rats by melatonin (MEL) after GMH. (**a**) Brain atrophy (percent tissue loss), (**b**) splenic weight, and (**c**) cardiac weight were measured at 4 weeks after injury. Values expressed as mean ± SEM, $n = 8$ (per group), $^*P < 0.05$ compared with controls (sham and needle trauma)

Melatonin is a widely tested neuroprotectant shown to ameliorate brain injury in adult animal models of cerebral ischemia [45] and hemorrhage [11]. This study supports the notion that the application of melatonin has no adverse affects in neonatal rats and can lead to improvements in functional outcomes after brain injury from hemorrhagic stroke in premature infants as well.

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Conflict of interest statement We declare that we have no conflict of interest.

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