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Preventive Effects of Melatonin Against Post-Traumatic Contusional Expansion in Rats

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ABSTRACT

AIM: To provide insight into the molecular mechanism of contusional expansion (CE) by creating experimentally induced contusion cerebri (CC) in rats and investigating whether melatonin administration prevents CE.

MATERIAL and METHODS: Rats were randomized into four groups: Group 1 (control, n=5), group 2 (trauma, n=25), group 3 (trauma plus placebo, n=25), and, group 4 (trauma plus melatonin, n=25). Rats in the control group were sacrificed without undergoing any invasive procedure. Groups 2, 3, and 4 were further divided into 5 subgroups (A–E), with animals in each sacrificed at 12, 24, 72, 120, and 168 h after CC induction. Samples from these subgroups were analyzed for levels of caspase 3, caspase 8, and matrix metalloproteinase-9, as well as for evidence of ischemia, blood-brain barrier (BBB) breakdown, vasogenic edema (VE), and hemorrhage. Temporal progression of CE and correlations between these variables were also investigated.

RESULTS: Our results indicated that the ischemia, BBB breakdown, and VE are early events that initiate CE, with VE and hemorrhagic transformation due to BBB breakdown identified as key factors. Melatonin treatment prevented CE injury.

CONCLUSION: Melatonin, a safe and well-tolerated substance with minimal toxicity, may serve as a potential therapeutic agent for preventing CE injury.

KEYWORDS: Melatonin, Blood-brain barrier, Contusional expansion, Matrix metalloproteinase-9, Caspases

INTRODUCTION

Contusional expansion (CE) is a significant cause of secondary injury and subsequent clinical deterioration (28). Studies have indicated that the progression of cerebral contusions occurs in approximately half of patients (25). Among the early pathological changes in CE, the breakdown of the blood-brain barrier (BBB) is considered a key event. Studies have suggested that increased BBB permeability leads to vasogenic edema (VE) and ultimately hemorrhagic transformation, both of which represent important stages in CE development (11,14).

Several studies have posited that impact injuries to the cortex result in mechanical injury tears and disrupt the brain parenchyma and blood vessels, resulting in a primary injury. This mechanical injury often leads to the structural failure of microvessels. The ensuing ischemia, driven by microvascular dysfunction and primary brain parenchymal damage, can trigger several pathways, including activating apoptotic effectors such as caspase 3 (C3), caspase 8 (C8), and the proteolytic enzyme matrix metalloproteinase-9 (PP9). These processes contribute to two pathological outcomes. First, they result in more BBB disruption. Second, they result in increased VE, accompanied by hemorrhagic progression in the contusion area. Together, these factors drive the growth of the primary lesion, otherwise known as CE (2,3,8,9,10).

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The pathophysiology of CE injury is a complex process, and research has been unable to elucidate the underlying molecular mechanisms (10). Contemporary therapeutic interventions for CE are predominantly focused on reducing the intracranial hypertension associated with CE and are not focused on its prevention. This limits their clinical utility (12,14,16,25).

Melatonin has demonstrated the ability to cross morphophysiological barriers such as the BBB. It is considered safe, even at higher concentrations, and is well-tolerated by humans with minimal toxicity. As a multifunctional molecule, melatonin may be a useful therapeutic agent for the treatment of central nervous system injuries (20,26,27). Melatonin has also been reported to attenuate cerebral edema in a controlled cortical impact mouse model by inhibiting MMP9 (26) and reducing apoptotic cell death via C3 suppression in a rat subarachnoid hemorrhage model (4). Other studies have shown that melatonin inhibits MMP9 activity, subsequently decreasing the risk of hemorrhagic transformation following cerebral ischemia-reperfusion and traumatic spinal cord injury (13).

Given these previous findings, the present study was designed with two primary objectives. First, to evaluate the molecular pathophysiology underlying the hemorrhagic transformation associated with CE, and second, to investigate the effect of melatonin on this process.

MATERIAL and METHODS

This study was carried out in the Experimental Research Laboratory of the Inonu University Faculty of Medicine, complying with the approval of the ethic committee and the guidelines for care and use of experimental animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

The experiments were performed on 15-week-old 80 Wistar albino female rats, weighing between 200–250 g. The animals were kept under standard conditions, including a 12 h light/dark cycle, a constant ambient temperature of 20 °C, and humidity maintained between 40–60%. The rats had free access to standard dry pellets and tap water throughout the study. Rats were randomly divided into four groups: Group 1 (control, n=5), group 2 (trauma, n=25), group 3 (trauma plus placebo, n=25), and, group 4 (trauma plus melatonin, n=25). All rats were fasted one day before surgery and pre-treated with enrofloxacin (2.27 mg/kg subcutaneously, Bayer, Germany). The rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) before surgery. All rats were placed on a heated surgical table to maintain a body temperature of 37°C. Supplemental doses of ketamine were provided as needed to maintain anesthesia.

The control group were sacrificed without undergoing any surgical procedure. The rats in groups 2, 3, and 4 underwent a standardized surgical procedure. The surgical area was shaved and cleaned antiseptically. A midline longitudinal incision was made on the scalp, and the underlying periosteum and muscles were dissected to expose the skull. A craniotomy (10 mm x 15 mm) was performed over the right parietal bone using a dental drill. To create the brain injury, the weight drop technique modified by Feeney et al. was used (7). A 9 g weight was dropped from a height of 50 cm onto a 10 mm diameter piston resting on the exposed dura, delivering an impact force of 450 g/cm.

To explore the temporal profile of the data, rats in groups 2, 3, and 4 were further divided into 5 subgroups (A–E), each comprising 5 rats. The subgroups were sacrificed at specific time points after injury: Subgroup A was sacrificed at 12 h, subgroup B was sacrificed after 24 h, subgroup C was sacrificed after 72 h, subgroup D was sacrificed after 120 h, and subgroup E was sacrificed after 168 h. Six hours after the procedure, intraperitoneal administration of 1 ml 2.5% alcohol or melatonin (purchased from Sigma; 20 mg/kg/day in 1 ml of 2.5% alcohol solution, warmed to 37 °C) was initiated in groups 3 and 4, respectively. Group 2, the trauma group, received no medication. Intraperitoneal melatonin or alcohol injections were continued until the time of sacrifice.

At the end of the specified period for each subgroup, the animals were re-anesthetized, and the ascending aorta was cannulated retrogradely through a thoracotomy. The craniocervical circulation was perfused with 200 ml of heparinized isosmotic phosphate buffer saline (0.1M, pH 7.4) at a physiological mean arterial pressure (80–90mmHg) using a peristaltic pump (May=PRS9508=991129-1). This was followed by perfusion with 200 ml of 0.1M phosphate buffer saline containing 4% paraformaldehyde. Brains were removed and the right and left hemispheres were separated. The right hemispheres, which contained the contusion epicenter, were post-fixed in 4% formalin and processed for paraffin embedding. Representative sections were sliced into 5 µm thick sections. A series of adjacent sections were used so that multiple histological and immunological markers could be examined in individual animals. The sections were also stained with hematoxylin-eosin (H&E) to enable evaluation of the degree of BBB breakdown, VE, and hemorrhage. For immunohistochemistry evaluation, immediately adjacent sections were processed simultaneously and stained with antibodies against C3, C8, and MMP9 (Abcam, polyclonal antibody for rats). These slides were then assessed by a neuropathologist. The number of positively stained cells was counted in 10 different sites, and the mean value was recorded.

VE results from dysfunction in the BBB. Both conditions are characterized by the movement of intravascular contents from the vasculature into the extracellular space and by the enlargement of the extracellular spaces (6). Therefore, we assessed both BBB breakdown and VE by measuring the extent of extracellular space enlargement, recorded as VE. A neuropathologist, blinded to group assignments, evaluated the edema on H&E-stained slides using a light microscope (Olympus, BX50, Tokyo, Japan). If the enlargement was minimal, VE was classified as grade 1. If the enlargement was moderate, VE was classified as grade 2. If the enlargement was severe, VE was classified as grade 3

The H&E-stained slides were assessed by a neuropathologist to evaluate the extent of hemorrhage and ischemia using a light microscope (Olympus, BX50, Tokyo, Japan). If the hemorrhage involved less than one-third of the high-power

field (HPF; x40 objectives), it was classified as grade 1. If hemorrhage involved between one-third to two-thirds of the HPF, it was classified as grade 2. If the hemorrhage involved over two-thirds of the HPF, it was classified as grade 3. To determine the extent of ischemia, the number of red neurons, which are typically observed in early ischemic damage, were used. Quantification of neuronal damage was performed by counting the number of pink acidophilic dead neurons (red neurons), following the method described by Kaku et al. (18). Red neurons were counted in 10 different HPFs (x40 objective) and the average number was calculated. If the number of red neurons was between 1–3, the degree of ischemia was classified as grade 1. If the number of red neurons was between 3–5, the degree of ischemia was classified as grade 2. If the number of red neurons was above 5, the degree of ischemia was classified as 3.

Statistical Analysis

The data from the control group were considered as the baseline values for all parameters. Data normality was evaluated using the Shapiro-Wilk test. Data were summarized as median (minimum-maximum) values. Group comparisons were performed using the Kruskal-Wallis test. When significant differences were detected, pairwise comparisons were performed using the Conover test. Statistically different groups, based on these pairwise comparisons, were indicated by different superscripts. For intra-temporal comparisons, a, b, and c superscripts were used, while x and y superscripts were used for comparisons between groups at each time point. Relationships between the variables across time points were evaluated using Spearman's rank correlation coefficient. A two-sided significance level of 0.05 was used for all analyses.

RESULTS

The median number of C3-positive cells was 0 (range = 0–0) in the control group. In groups 2 (trauma) and 3 (trauma plus placebo), the median C3-positive cell counts were 8 (6–10) and 8 (6–10) at 12 h, 15 (11–18) and 13 (11–17) at 24 h, 15 (12–17) and 15 (11–17) at 72 h, 9 (6–9) and 8 (5–10) at 120 h and, 2 (1–5) and 2 (1–4) at 168 h, respectively. In both groups, C3 levels began to increase immediately after trauma, peaked between 24 and 72 h, and significantly decreased thereafter ($p<0.05$). Significant differences were observed across all time points within these groups. C3 levels were significantly elevated in these groups across all time points compared to the control group ($p<0.05$), with no significant difference between groups 2 and 3. In group 4 (trauma plus melatonin), the median C3-positive cell counts were 3 (1–5), 7 (6–8), 7 (6–8), 4 (1–5), and 2 (1–3) at 12, 24, 72, 120, and 168 hours, respectively. Although group 4 followed a similar temporal pattern to groups 2 and 3, C3 levels in group 4 were significantly lower than in groups 2 and 3 at all time points except 160 h ($p<0.05$; Figure 1).

The median number of C8-positive cells was 0 (0–0) in the control group. In groups 2 and 3, the median C8-positive cell counts were 9 (6–10) and 9 (8–10) at 12 h, 13 (11–16) and 15 (12–15) at 24 h, 9 (6–10) and 9 (7–9) at 72 h, 8 (7–10) and 8 (7–10) at 120 h, and 3 (2–5) and 3 (2–5) at 168 h, respectively. In these groups, C8 began to increase immediately after trauma and continued to significantly increase until peaking at 24 h ($p<0.05$). In contrast to C3 levels, the C8 levels began to decrease immediately after peaking and continued to decline significantly up to 72 h post-injection ($p<0.05$). From 72 to 120 h, the decline slowed and remained relatively stable, followed by a further significant decrease after 120 h ($p<0.05$). C8 levels were significantly increased in groups 2 and 3 at all time points compared to the control group ($p<0.05$). However, no significant difference was observed between groups 2 and 3. In group 4, the median C8-positive cell counts were 2 (1–5), 6 (6–8), 7 (6–7), 2 (1–5), and 1 (1–2) at 12, 24, 72, 120, and 168 hours, respectively. C8 levels in group 4 were significantly lower than those in groups 2 and 3 at all time points ($p<0.05$). However, in contrast to C3, C8 levels in group 4 did not show a similar temporal pattern to groups 2 and 3 (Figure 2).

The median number of MMP9-positive cells was 0 (0–0) in the control group. In groups 2 and 3, the median MMP9-positive cell counts were 8 (6–10) and 8 (7–10) at 12 h,

13 (12–16) and 14 (12–16) at 24 h, 15 (13–18) and 15 (13–17) at 72 h, 9 (8–10) and 9 (8–10) at 120 h, and 9 (8–10) and 8 (8–9) at 168 h, respectively. In both groups, MMP9 levels showed a similar pattern to C3 levels. They began to significantly

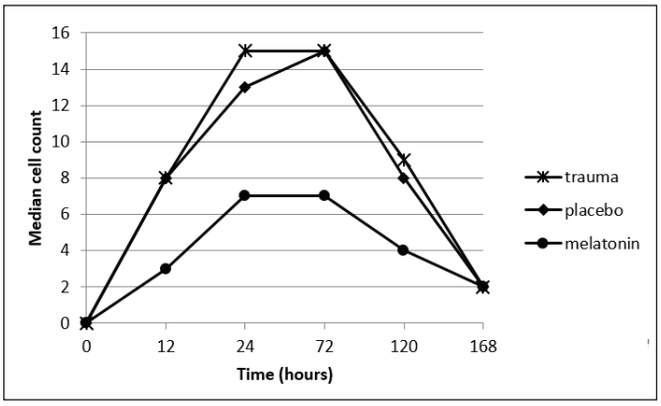

Figure 1: The temporal course of caspase 3 (C3) levels in the contusion areas.

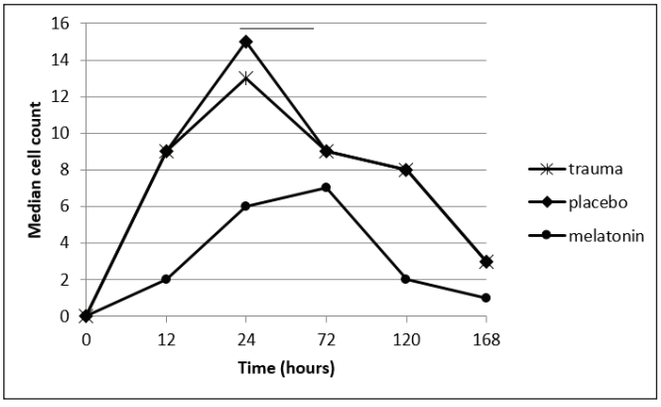

Figure 2: The temporal course of caspase 8 (C8) levels in the contusion areas.

increase immediately after trauma, peaking between 24 h and 72 h ($p<0.05$). A significant decline followed up to 120 h, after which levels remained stable. MMP9 levels were significantly increased in groups 2 and 3 at all time points compared to the control group ($p<0.05$), with no significant differences between groups 2 and 3. In group 4, the median MMP9-positive cell counts were 2 (1–3), 7 (6–8), 7 (6–8), 2 (1–3), and 2 (1–3) at 12, 24, 72, 120, and 168 hours, respectively. While there was no significant difference in MMP9 levels between 120 and 168 h, significant differences were observed between the other time points ($p<0.05$). MMP9 levels in group 4 were significantly lower compared to those in groups 2 and 3 at all time points ($p<0.05$; Figure 3).

The microscopic image of red neurons in the contusion area is shown in Figure 4. The median number of red neurons indicating ischemia was 0 (0–0) in the control group. In groups 2 and 3, the median red neuron counts were 10 (7–18) and 10 (8–17) at 12 h, 15 (5–18) and 14 (5–16) at 24 h, 15 (13–18) and 15 (14–17) at 72 h, 16 (14–18) and 15 (14–18) at 120 h, and 16 (14–17) and 16 (15–18) at 168 h, respectively. Ischemia levels were significantly higher in these groups compared to the control group at all time points ($p<0.05$); however, there was no significant difference between the groups at any time points. Ischemia levels started to increase immediately after trauma, showing an initial peak at 12 h and a second peak at 24 h ($p<0.05$). Following this, levels continued to increase more gradually. In group 4, the median red neuron counts were 6 (6–7), 5 (4–5), 7 (6–8), 7 (6–8), and 6 (5–8) at 12, 24, 72, 120, and 168 hours, respectively. Ischemia levels were significantly lower in group 4 compared to groups 2 and 3 at all time points ($p<0.05$; Figure 5).

Microscopic images of VE and red neurons in the contusion area are shown in Figure 4. The median grade of VE was 0 (0–0) in the control group. In groups 2 and 3, the median grades were 1 (0–2) and 1 (0–2) at 12 h, 1 (1–2) and 1 (1–2) at 24 h, 2 (1–2) and 2 (1–2) at 72 h, 1 (1–1) and 1 (1–1) at 120 h, and 1 (1–1) and 1 (1–1) at 168 h, respectively. In these groups, a significant increase in VE was observed at time points compared to the control group ($p<0.05$). However, there were no significant differences between groups 2 and 3. VE and BBB breakdown started to increase immediately after trauma, with an initial peak at 12 h. Their development then remained unchanged until 24 h, with a second peak then observed at 72 h. Following this, levels began to decrease until 120 h, at

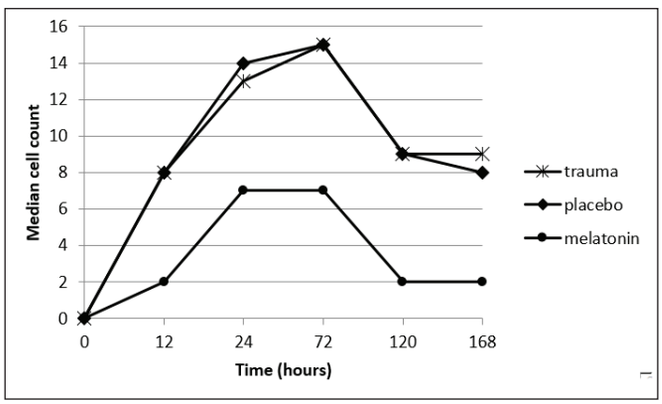

Figure 3: The temporal course of matrix metalloproteinase 9 (MMP9) levels in the contusion areas.

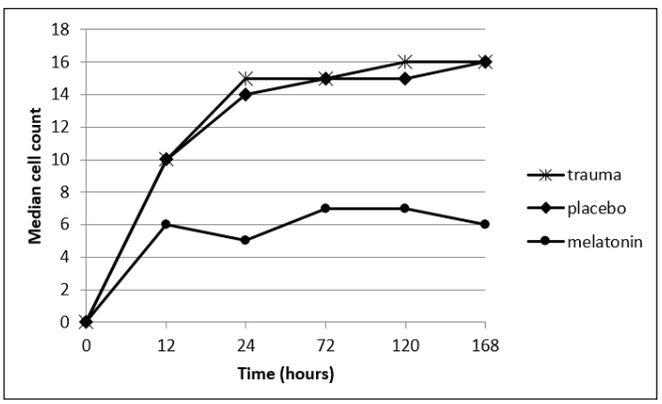

Figure 5: The temporal course of ischemia in the contusion areas.

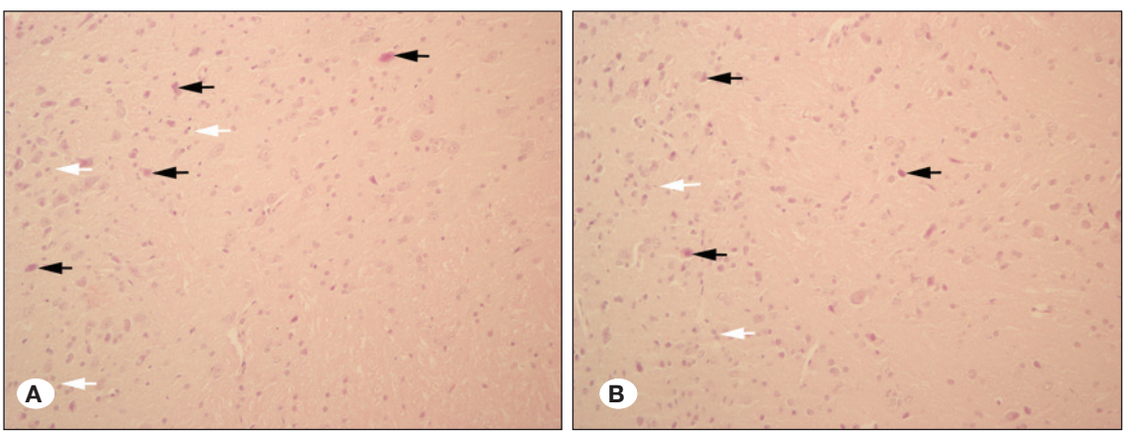

Figure 4: A microscopic image depicting ischemia, vasogenic edema (VE), and blood-brain barrier (BBB) breakdown, as well as red "ischemic" neurons in the contusion area. The enlargement of the extracellular spaces is a marker of VE. Red "ischemic" neurons are indicated by black arrows and glial proliferation by white arrows. **A)** Trauma group. **B)** Trauma plus melatonin group. HE, x 90.

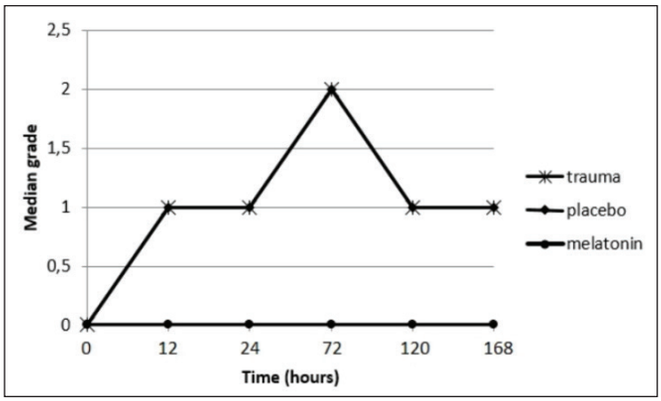

Figure 6: The temporal course of vasogenic edema (VE) in the contusion areas.

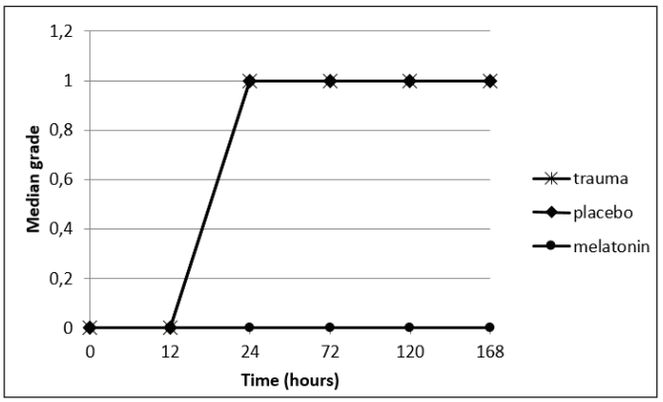

Figure 8: The temporal course of hemorrhagic transformation in the contusion areas.

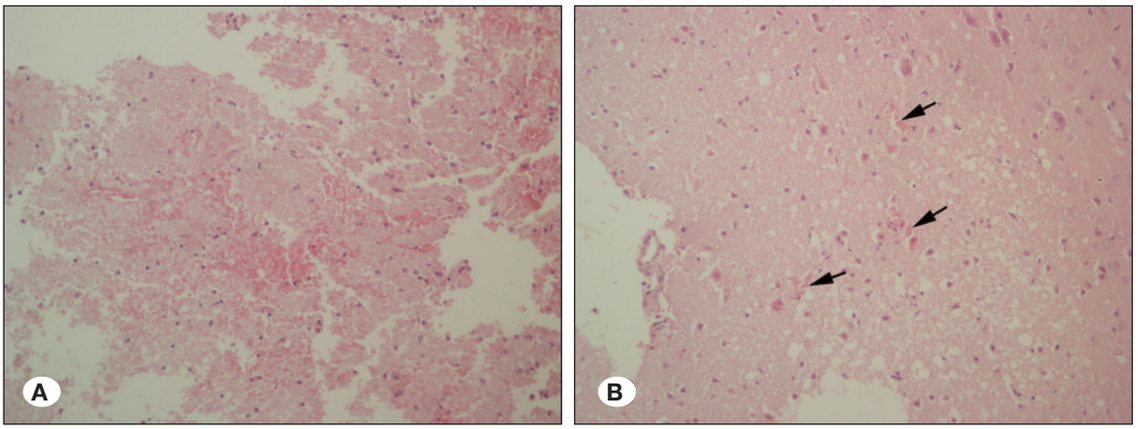

Figure 7: A microscopic image of hemorrhage in the contusion area. **A)** Trauma group; severe hemorrhage. **B)** Trauma plus melatonin group; minimal hemorrhage, "black arrows". HE, x 90.

which point they remained stable. In group 4, the median VE grades were 0 (0–1), 0 (0–1), 0 (0–1), 0 (0–1), and 0 (0–1) at 12, 24, 72, 120, and 168 hours, respectively. Although there were no significant differences between time points within group 4, the levels of VE were markedly lower than in groups 2 and 3 across all time points (Figure 6).

Microscopic images of hemorrhage in the contusion area are shown in Figure 7. The median hemorrhage grade was 0 (0–0) in the control group. In groups 2 and 3, the median hemorrhage grades were 0 (0–1) and 0 (0–0) at 12 h, 1 (0–1) and 1 (0–1) at 24 h, 1 (1–2) and 1 (1–1) at 72 h, 1 (1–2) and 1 (1–2) at 120 h, and 1 (1–1) and 1 (1–1) at 168 h, respectively. No hemorrhagic progression was observed before 12 h in these groups. However, the level of hemorrhagic progression was significantly higher at 24, 72, 120, and 168 h in these groups compared to both the control group and 12 h subgroups ($p<0.05$). No significant difference was observed between groups 2 and 3. Hemorrhage levels started to increase 12 h after trauma, peaking at 24 h and then stabilizing. In group 4, the median hemorrhage grades were 0 (0–1), 0 (0–1), 0 (0–1), 0 (0–0), and 0 (0–0) at 12, 24, 72, 120, and 168 hours, respectively, indicating an absence of any substantial disparities among the subgroups in group 4. The level of hemorrhagic progression in group 4 was significantly lower than in groups 2 and 3 ($p<0.05$; Figure 8).

Table I illustrates the intercorrelations among parameters within specified time intervals. Between 0 h and 12 h, significant positive correlations were observed between levels of C3 and C8 (0.724, $p<0.001$), C3 and MMP9 (0.943, $p<0.001$), and C8 and MMP9 (0.702, $p<0.001$). Between 12 h and 24 h, positive correlations were observed between levels of C3 and ischemia (0.468, $p=0.009$), C8 and MMP9 (0.493, $p=0.006$), hemorrhage and VE (0.633, $p<0.001$), and hemorrhage and MMP9 (0.389, $p=0.034$). Between 24 h and 72 h, negative correlations were observed between levels of C3 and hemorrhage (–0.396, $p=0.030$) and C8 and MMP9 (–0.395, $p=0.031$), while positive correlations were observed between C3 and MMP9 (0.550, $p=0.002$), hemorrhage and VE (0.370, $p=0.044$), and VE and MMP9 (0.437, $p=0.016$). Between 72 h and 120 h, negative correlations were observed between C3 and C8 (–0.455, $p=0.012$), C3 and ischemia (–0.537, $p=0.002$), C8 and MMP9 (–0.544, $p=0.002$), C8 and VE (–0.469, $p=0.009$), and VE and ischemia (–0.509, $p=0.004$), while positive correlations were observed between C8 and ischemia (0.553, $p=0.002$) and hemorrhage and VE (0.399, $p=0.029$). Between 120 h and 168 h, positive correlations were observed between

Table I: The Correlations Among Parameters in Intervals at All Time

0 h – 12 h	**C 8**	**Hemorrhage**	**VE**	**Ischemia**	**MMP9**
C 3	**0.724 (p<0.001)**	0.058 (p=0.760)	0.001 (p=0.994)	-0.078 (p=0.683)	**0.943 (p<0.001)**
C 8	1.000	0.176 (p=0.352)	0.040 (p=0.837)	-0.196 (p=0.300)	**0.702 (p<0.001)**
Hemorrhage		1.000	0.056 (p=0.774)	-0.250 (p=0.183)	0.117 (p=0.537)
VE			1.000	0.056 (p=0.774)	0.161 (p=0.405)
Ischemia				1.000	-0.020 (p=0.918)
12 h – 24 h					
C 3	-0.290 (p=0.121)	0.020 (p=0.918)	-0.133 (p=0.484)	**0.468 (p=0.009)**	-0.099 (p=0.605)
C 8	1.000	0.161 (p=0.394)	0.010 (p=0.957)	-0.150 (p=0.429)	**0.493 (p=0.006)**
Hemorrhage		1.000	**0.633 (p<0.001)**	0.357 (p=0.053)	**0.389 (p=0.034)**
VE			1.000	0.105 (p=0.580)	-0.020 (p=0.918)
Ischemia				1.000	0.128 (p=0.499)
24 h – 72 h					
C 3	-0.042 (p=0.825)	**-0.396 (p=0.030)**	0.123 (p=0.518)	0.197 (p=0.296)	**0.550 (p=0.002)**
C 8	1.000	-0.315 (p=0.090)	-0.114 (p=0.547)	-0.016 (p=0.934)	**-0.395 (p=0.031)**
Hemorrhage		1.000	**0.370 (p=0.044)**	-0.289 (p=0.121)	0.002 (p=0.993)
V E			1.000	-0.068 (p=0.721)	**0.437 (p=0.016)**
Ischemia				1.000	-0.066 (p=0.730)
72 h – 120 h					
C 3	**-0.455 (p=0.012)**	-0.301 (p=0.106)	0.184 (p=0.330)	**-0.537 (p=0.002)**	-0.002 (p=0.992)
C 8	1.000	-0.174 (p=0.357)	**-0.469 (p=0.009)**	**0.553 (p=0.002)**	**-0.544 (p=0.002)**
Hemorrhage		1.000	**0.399 (p=0.029)**	-0.057 (p=0.763)	0.302 (p=0.105)
V E			1.000	**-0.509 (p=0.004)**	0.303 (p=0.104)
Ischemia				1.000	-0.148 (p=0.435)
120 h – 168 h					
C3	0.314 (p=0.092)	0.229 (p=0.223)	0.256 (p=0.172)	**0.438 (p=0.015)**	-0.062 (p=0.745)
C8	1.000	**0.390 (p=0.033)**	-0.278 (p=0.137)	**0.441 (p=0.015)**	-0.343 (p=0.063)
Hemorrhage		1.000	0.000 (p=1.000)	**0.447 (p=0.013)**	-0.174 (p=0.359)
VE			1.000	0.000 (p=1.000)	0.000 (p=1.000)
Ischemia				1.000	0.342 (p=0.065)

C3: *Caspase3,* ***C8:*** *Caspase8,* ***MMP9:*** *Matrix metalloproteinase-9,* ***VE:*** *Vasogenic edema and blood-brain barrier breakdown,* ***h:*** *Hour.*

C3 and ischemia (0.438, p=0.015), C8 and hemorrhage (0.390, p=0.033), C8 and ischemia (0.441, p=0.015), and hemorrhage and ischemia (0.447, p=0.013).

DISCUSSION

VE and hemorrhagic transformation are progressive processes and major causes of CE injury (12,14,18). Studies have indicated that VE formation begins immediately after trauma and typically peaks between 48 h and 72 h, while hemorrhagic progression most frequently occurs between the first 12 h and 24 h, peaking at 24 h (1,12,16,24,28). Our data indicated that no hemorrhagic progression occurred before 12 h post-injury, while VE, BBB breakdown, and ischemia levels began to increase immediately after trauma. Both VE and BBB breakdown levels initially peaked after 12 h, stabilized until 24 h, and had a second peak at 72 h. After this, they began to decrease until 120 h. The present findings lend further support

to previous studies, demonstrating that VE formation begins immediately following trauma and typically peaks between 48 h and 72 h, while hemorrhagic progression typically occurs between 12 h and 24 h, peaking at 24 h (1,12,16,24).

It is well established that the initial and most important contributors to BBB breakdown and ischemia after traumatic brain injury (TBI) are mechanical tears and shearing forces impacting the brain parenchyma and blood vessels (1,6,18,24). The initiation of BBB breakdown and subsequent ischemia triggers the activation of multiple pathways, including the activation of the apoptotic effectors C3 and C8 and the proteolytic enzyme MMP9. This results in further BBB disruption, subsequently increasing VE and promoting hematoma progression. This cascade leads to further expansion of the primary lesion (14,16,22,28).

Our study showed that C3, C8, and MMP9 levels increased alongside VE and BBB breakdown between 0 h and 12 h post-injury. Positive correlations were observed between levels of C3 and C8, C3 and MMP9, and C8 and MMP9 in this period. However, no significant correlations were observed between VE, ischemia, or hemorrhage, or between these factors and C3, C8, and MMP9 levels within the same interval. These findings indicate that C3, C8, and MMP9 are co-activated immediately after trauma and operate through interconnect pathways. These findings also support previous studies indicating that early edema, BBB breakdown, and ischemia following a TBI are direct consequences of mechanical tissue and vascular injury (14,16,22,28).

Our data indicated that between 12 h and 24 h post-injury, C3, C8, and MMP9, and ischemia levels continued to increase, while VE and BBB breakdown levels stabilized. This interval coincides with the onset and peak of hemorrhagic progression. Notably, correlations were observed between C3 and ischemia, C8 and MMP9, hemorrhagic progression and MMP9, hemorrhagic progression and VE, and hemorrhagic progression and BBB breakdown However, the correlation between hemorrhagic progression and MMP9 levels was lower than the correlation between hemorrhagic progression and VE and between C8 and MMP9.

These findings support those of previous studies, suggesting that apoptotic factors may contribute to microvascular disruption and exacerbate BBB damage as part of the secondary injury cascade (2,3,8,9,10,30). Furthermore, our findings highlight BBB breakdown and VE as central events driving hemorrhagic progression in CE. MMP9 likely plays a key role in initiating hemorrhagic transformation by increasing BBB breakdown and VE beyond a critical threshold level. This is presumably mediated by MMP9's capacity to degrade components of the basal lamina and disrupt tight junction proteins in both injured and intact vessels (23,29).

Our data demonstrated that between 24 h and 72 h, VE and MMP9 levels continued to rise, C8 levels started to decrease, and C3, ischemia, and hemorrhage levels remained unchanged. During this interval, negative correlations were observed between C3 and hemorrhagic progression and between C8 and MMP9, while positive correlations were observed between VE and hemorrhagic progression and VE and MMP9. These findings suggest that while MMP9 plays a critical role in exacerbating BBB breakdown and VE, other factors likely play additional roles in modulating these processes (10,14,22).

In the interval between 72 h and 120 h, C3, VE, and MMP9 levels started to decrease significantly, while the decline in C8 levels slowed until levels were near stable. Ischemia and hemorrhage levels also remained unchanged. Negative correlations were observed between C3 and C8, C3 and ischemia, C8 and edema, C8 and MMP9, and edema and ischemia. In contrast, positive correlations were observed between edema and hemorrhagic progression and C8 and ischemia. These findings suggest that endothelial apoptotic damage mediated by C8 persisted during this time. The positive correlation seen between C8 and ischemia may reflect the pro-inflammatory effects of C8 (19).

In the interval between 120 h and 168 h, C3 and C8 levels continued to decrease, ischemia levels continued to rise slowly, and MMP9, ischemia, VE, and hemorrhage levels remained unchanged. Positive correlations were observed between C3 and ischemia, C8 and hemorrhagic progression, C8 and ischemia, and between hemorrhagic progression and ischemia. These findings suggest that endothelial apoptotic damage continued to contribute to ischemia during this period. Additionally, hemorrhagic progression may have influenced ischemic progression, likely due to its mass effect (21).

In the current study, melatonin administration completely prevented VE and hemorrhagic transformation (i.e., CE) by reducing C3, C8, MMP9, and ischemia levels across all time points, except for 120 and 168 h post-injury in the case of MMP9. However, an incontinency was observed between the reduction rates of C3, C8, and MMP9 and those of VE and hemorrhagic progression, with C3, C8, and MMP9 levels decreasing partially but significantly, while VE and hemorrhagic progression levels decreased almost completely. This discrepancy may be explained by the involvement of additional pathways beyond C3, C8, and MMP9 in the development of CE, which melatonin may also inhibit (5,10,14,15,22).

In contrast to the near-complete reduction of VE and hemorrhage levels, melatonin administration only reduced ischemia levels partially, similar to its impact on C3, C8, and MMP9 levels. This may be attributed to continued primary vascular dysfunction (17). These findings support previous studies that suggested that the structural failure of microvessels caused by primary injuries triggers multiple pathways, ultimately leading to CE through increased VE and hemorrhagic progression in the primary lesion (2,3,8,9,10).

Taken together, the results of our study imply that:

1. CE is driven by a complex interplay of molecular, cellular, structural, and functional changes, including microcirculation dysfunction, ischemia, BBB breakdown, edema, and hemorrhage arising from both primary and secondary injuries.

2. BBB breakdown leads to VE and hemorrhagic transformation, which are key factors in CE development. VE formation begins immediately after trauma and follows a biphasic pattern (first peak at 12 h, second peak at 72 h), while hemorrhagic progression starts at 12 h and peaks at 24 h.
3. Ischemia, BBB breakdown, and VE are the initial changes that trigger CE. Hemorrhagic transformation occurs later once VE exceeds a certain threshold and is further promoted by MMP9, consequently exacerbating CE.
4. While C3, C8, and, in particular, MMP9 play key roles in CE pathogenesis, additional pathways are likely to contribute to its development.
5. Melatonin effectively prevented CE by inhibiting or modulating several key pathways involved in VE development and hemorrhagic transformation, supporting its potential use as a multifunctional therapeutic agent.

The current study has several limitations. First, we only investigated temporal changes in CE, not spatial changes. Second, we did not examine all potential factors or pathways that may influence CE. Further studies are needed to explore these potential factors and pathways, including their spatiotemporal distributions and interactions. Finally, BBB breakdown was assessed indirectly, with VE levels used as a surrogate marker.

CONCLUSION

The present study demonstrated that melatonin inhibits CE by preventing BBB breakdown and VE, which are key events that lead to hemorrhagic progression, and by ameliorating ischemia. These findings suggest that melatonin, a safe and well-tolerated molecule with minimum toxicity in humans, could be utilized as a potential therapeutic agent for CE prevention. However, further studies are required to validate this hypothesis.

Declarations

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AUTHORSHIP CONTRIBUTION

Study conception and design: MAA, CG, RP

Data collection: MAA, CG, RP, HG

Analysis and interpretation of results: HG

Draft manuscript preparation: MAA

Critical revision of the article: MAA

Other (study supervision, fundings, materials, etc...): MAA, CG, RP, HG

All authors (MAA, CG, RP, HG) reviewed the results and approved the final version of the manuscript.

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