Original Article

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Repositioning of an anti-depressant drug, agomelatine as therapy for brain injury induced by craniotomy

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Summary **Traumatic brain injury (TBI) leads to the disruption of blood-brain barrier integrity and therefore results in increased brain water content (brain edema). Brain edema is a significant factor for increased intracranial pressure (ICP), which ultimately causes functional disability and death. The decompressive craniotomy (DC) is a surgical procedure widely used for treating increased ICP following TBI. The life-saving craniotomy itself results in brain injury. The objective of this study is to investigate the effect of agomelatine against craniotomy induced brain injury. The craniotomy was performed by a variable speed micro-motor dental driller of 0.8 mm drill bit. The present study, in addition to blood-brain permeability, brain water content (edema) and histological examination of the brain, also estimated locomotor activity, oxidant, and antioxidant parameters. Results show that the craniotomy induced increase in the blood-brain barrier permeability, brain water content (edema), oxidative stress (lipid peroxide and nitric oxide) and impaired antioxidant mechanisms (superoxide dismutase, catalase, and reduced glutathione) in rats. The craniotomy was also found to increase neuronal cell death indicated by augmented chromatolysis and impaired locomotor activity. Administration of agomelatine after the craniotomy ameliorated histopathological, neurochemical and behavioral consequences of craniotomy. Thus agomelatine is effective against brain injury caused by craniotomy.**

Keywords: Traumatic brain injury, craniotomy, agomelatine, blood-brain barrier permeability, brain edema, intracranial pressure

1. Introduction

Traumatic brain injury (TBI) is defined as an impairment of brain functions caused by an external mechanical impact (*1*). The mechanical impacts on the brain in TBI cause fracture of cranial vault bone, which sometimes depresses into the brain, leads to intracranial bleeding and hemorrhage within the brain parenchyma (*2*). This damage results in extracellular water accumulation due to blood-brain barrier disruption, sustained intracellular water collection, osmotic imbalances between blood and tissue, and obstruction of cerebrospinal fluid outflow. This leads to vasogenic, cytotoxic/cellular, osmotic, and hydrocephalic/interstitial edema, respectively (*3*). Cerebral swelling due to edema results in the increased intracranial pressure (ICP) and the brain herniation, followed by coma and death (*4*).

The decompressive craniectomy (DC) is a widely used neurosurgical treatment for increased ICP following TBI (*5*). In DC, a part of the skull is removed to allow the swell brain to expand without being squeezed. DC caused brain injury, revealed by morphological, behavioral, and biochemical changes (*6*). Thus DC reduces the mortality, however, it converts fatality into survival with severe disability (*7*).

Brain injury results in decrease in the levels of melatonin receptors subtype 1 (MT_1) and 2 (MT_2) (*8*). An earlier report shows that melatonin exerts neuroprotective effects against brain injury induced by trauma (*9*) as well as hypoxic-ischemia (*10*). Melatonin attenuates TBI induced inflammation (*11*) and preserves the blood-brain barrier integrity and permeability *via*

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matrix metalloproteinase-9 inhibition (*12*). Additional studies using different experimental TBI models showed that melatonin decreases brain edema, blood-brain barrier permeability, ICP, and neuronal cell death probably due to inhibition of oxidative stress and the attenuation of nuclear factor-kappaB (NF-kappaB) (*13-15*). MT_1, MT_2, and melatonin receptor subtype 3 (MT_3) melatonin receptors had anti-edema effects while MT_1 and MT_2 have a role in protecting the blood-brain barrier (*16*).

Agomelatine is the first melatonergic antidepressant. It is a potent agonist of the melatonin receptors MT_1 and MT_2 and an antagonist at serotonin-2C ($5\text{-}HT_{2C}$) receptors (*17*). Agomelatine enhances adult hippocampal neurogenesis and increases the expression of several molecules associated with neuroplasticity (*18,19*). The action of agomelatine on neurogenesis is likely to reside in its antagonism to the $5HT_{2C}$ receptor (*20*). However, until now agomelatine has not been explored for its neuroprotective activity against craniotomy induced brain injury. In this study, we hypothesized that agomelatine might help in faster recovery after craniotomy *via* synergistic agonistic action on MT_1/MT_2 and antagonist on $5\text{-}HT_{2C}$ receptors. Therefore, the present work evaluates the neuroprotective activity of agomelatine against craniotomy induced brain injury. In addition to brain edema and its histological examination, the present study also includes estimation of locomotor activity, blood-brain permeability, oxidant and antioxidant parameters.

2. Materials and Methods

2.1. *Animals*

Sprague Dawley (SD) rats (n = 90), weighing 250 ± 10 g, of either sex were used for the study. The animals were housed 3 per cage in polypropylene cages, and the environmental conditions of the animal room were as per a specific design. A 10% air exhaust in the air conditioning unit was maintained along with a relative humidity of 60 ± 5% and a temperature of 25 ± 3°C was stabilized. A 12 h light/dark cycle was also regulated for the experimental animals. Food and water provided *ad libitum* to the animals during the experimental period. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee before the initiation of the experiment.

2.2. *Drugs, reagents, and solvents*

Agomelatine (Agoviz) was procured from Abbott, Mumbai, India. Isoflurane and Evans blue were purchased from Raman and Weil Pvt. Ltd. (Mumbai, India) and Sigma Aldrich (Bangalore, India), respectively. Tris buffer, ethylenediaminetetraacetic acid (EDTA), Triton X 100, pyrogallol, thiobarbituric acid (TBA), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), naphthylenediamine dihydrochloride, sulphanilic acid, L-reduced glutathione were purchased from HiMedia laboratories Pvt. Ltd., Mumbai, India. All the other solvents and chemicals were purchased from Loba Chemie (Mumbai, Maharashtra, India).

2.3. *Craniotomy*

The craniotomy procedure was performed on rats as demonstrated by Cole *et al*. (*6*). Briefly, rats were anesthetized with 4% isoflurane and maintained with 2% in 98% oxygen. Once anesthetized, the animals were placed in a prone position with anesthesia delivered using nosecone. The animal's head and surgical site were sterilized with the application of 95% ethanol after shaving. A midline incision on the scalp exposed the skull. The craniotomy was performed by using a variable speed micro-motor dental driller of 0.8 mm drill bit. 2.5 mm radius bone flap above the right hemisphere was carefully drilled. Bone flap was removed, and the brain was exposed to the normal environment for 4 min while normal saline was continuously poured. After 4 min of exposure, the bone flap was replaced and secured with Ethicon bone wax. The incision was sutured, and Povidone Iodine solution was applied to the sutured site. Animals were returned to recovery cage with sodium lamp to provide heat and continuously monitored.

2.4. *Experimental design*

SD rats were divided into five groups with six animals each. All groups except normal were then subjected to craniotomy. Normal and craniotomy group rats received the 0.3% carboxymethylcellulose (CMC) (3 mL/kg, *p.o*.) suspension as the vehicle and other three groups received a single treatment of agomelatine (1, 3, and 10 mg/kg, *p.o*.) after one h of craniotomy. Alterations in the behavior of all the animals were assessed using the beam walking test, and pole test. All animals were sacrificed and brains were taken out for histopathology, estimation of brain water content, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase and reduced glutathione.

2.5. *Behavioral assessment*

All behavioral testing was carried out after 24 h of craniotomy and agomelatine treated animals in a sound proof room with subdued lighting.

2.5.1. *Beam walking test*

The beam-walk test was done to measure the loss of balance and coordination generally seen post-trauma (*21*). The surface of a 2.5 × 112 cm wooden beam was elevated 60 cm above the floor by the wooden support. A 20 × 25 × 24 cm goal box with a 10 cm opening was

Table1. Score for beam walking behavior of rat

Score	Observation
1	Rat is unable to place the affected hind paw on the horizontal surface of the beam
2	Rat places the affected hindpaw on the horizontal surface of the beam and maintains balance but is unable to traverse the beam
3	Rat traverses the beam dragging the affected hindpaw
4	Rat traverses the beam and once places the affected hindpaw on the horizontal surface of the beam
5	Rat crosses the beam and places the affected hindpaw on the horizontal surface of the beam to aid less than half its steps
6	Rat uses the affected hindpaw to aid more than half its steps
7	Rat traverses the beam with no more than two footsteps

placed at the one end of the beam. Two trials were given to each animal with 10 min interval. For each trial, the rat was placed at the start end of the beam opposite to the goal box. After two trials, the result was recorded. The performance was scored on a 7-point scale as mentioned in Table 1.

2.5.2. *Pole test*

Pole test was performed using the method described by Ogata *et al*. (*22*). The time for the rats to descend from the top of the roughly surfaced pole to the floor was recorded in all the animals of each group. Pole: 2.5 cm diameter, 100 cm height with 120 sec cut-off time.

2.6. *Estimation of oxidative stress*

2.6.1. *Assessment of lipid peroxidation*

MDA is one of the final products of the lipid peroxidation (LPO) in the cells. It was estimated as described in Draper and Hadley (*23*) with slight modification. Brain tissue was isolated and the cortex region was weighed 100 mg. Weighed tissue was transferred to tubes containing 5 mL Hanks' Balanced Salt Solution (HBSS) buffer and homogenized in 3 cycles of 30 sec each at 3,000 rpm with 30 sec gap. Tissue homogenate was centrifuged for 10 min at 3,000 rpm and 25°C. The supernatant was discarded and the cell pellet was taken. The cell pellet was transferred into tubes containing 0.2 mL of sodium dodecyl sulfate (SDS), 1.5 mL of acetic acid, 1.5 mL of TBA and 0.7 mL of MilliQ water. 0.1 mL HBSS was added in control tubes instead of homogenate. The tubes were kept in boiling water bath for 1 h. After boiling, 1 mL Milli Q was added to each tube. 5 mL butanol: pyridine (15:1) was added to each tube and vortexed for five mins. The organic layer was centrifuged at 3,000 rpm for 10 min at 25°C and the amount of MDA formed was measured by the absorbance of the upper organic layer at 532 nm. The concentration of MDA was calculated in µM/mg brain tissue using a standard curve prepared with 1,1,3,3-tetraethoxypropane (TEP).

2.6.2. *Assessment of nitric oxide*

The nitrite level is the marker of nitric oxide (NO). Nitrite level in brain tissues were estimated using the method described by Hevel and Marletta (*24*) with slight modification. Brain tissue was isolated and 100 mg cortex region was homogenized in 5 mL of ice-cold phosphate buffer at 3,000 rpm for 2 min in 2 cycles with 30 sec gap. In a separate tube containing freshly prepared 100 µL of Griess reagent and 2 mL of phosphate buffer, 300 µL of the homogenized solution was added. Tubes were incubated at room temperature for 30 min and absorbance was measured at 548 nm. The total nitrite concentration was calculated in mM/mg of tissue from a standard curve prepared with sodium nitrite.

2.6.3. *Assessment of superoxide dismutase (SOD)*

The SOD levels were estimated using the method described by Marklund and Marklund (*25*) with slight modification. Brain tissue was isolated, and 100 mg cortex region was homogenized in 4 mL Tris-EDTA (chilled) buffer by three cycles of 30 sec each at 3,000 rpm with 30 sec gap. In each tube, 1 mL of 1% Triton-X-100 was added. The tube was vortexed and incubated for 20 min at 4-8°C. The content was transferred to microcentrifuge tubes and centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was taken for the spectroscopic analysis. Zero time absorbance was taken at 420 nm followed by recording the absorbance reading every 60 sec for 10 min at 25°C. The above-mentioned reaction mixtures without the brain homogenate served as control. The rate of increase in absorbance units (A) per minute for the control and the test sample(s) was determined, and the percentage inhibition for the test sample(s) was calculated by the following formula:

$$\% \text{ inhibition} = \{[(\Delta A_{420}\ \text{nM/min})_{control} - (\Delta A_{420}\ \text{nM/min})_{test}]/(\Delta A_{420}\ \text{nM/min})_{control}\} \times 100$$

Units of the SOD activity were expressed as the amount of enzyme required to inhibit the reduction of pyrogallol by 50%, and the activity was expressed in units per g of brain tissue.

2.6.4. *Estimation of catalase*

The catalase levels were estimated using the method described by Sinha (*26*) with slight modifications. Brain tissue was isolated, and 100 mg cortex region was

homogenized in 5 mL phosphate buffer at 1,800 rpm in 3 cycles for 30 sec with 30 sec gap each. 1 mL tissue homogenate was diluted up to 5 mL using phosphate buffer and from diluted homogenate 1 mL was mixed with 2 mL of hydrogen peroxide (H_2O_2) (100 mM) and absorbance was noted for 0-10 min at 240 nm. Catalase activity was calculated per min per mg of brain tissue using the standard curve of H_2O_2.

2.6.5. *Estimation of reduced glutathione*

The reduced glutathione levels were estimated using the method described by Ellman (*27*) with slight modification. Brain tissue was isolated and 100 mg cortex region was homogenized in 5 mL of ice-cold phosphate buffer. In tissue homogenate, 0.1 mL of trichloroacetic acid was added. Centrifuged at 3,900 g at 25°C for 10 min. 1 mL of the supernatant was mixed with 1 mL DTNB and 1 mL phosphate buffer. It was vortexed for 1 min and incubated for 5 min at room temperature. Absorbance was noted at 412 nm.1 mL DTNB with 2 mL phosphate buffer was taken as blank. Reduced glutathione levels were calculated as µM/mg of brain tissue using a standard curve.

2.7. *Estimation of brain water content*

The brain water content was used to estimate edema, which forms as a consequence of the blood-brain barrier breakdown. Brain water content was estimated using the method described by Shigeno *et al*. (*28*) with slight modifications. Briefly, after decapitating the rats, the brains were removed. The injured hemisphere was weighed (wet weight), dipped in absolute alcohol for 30 min, dried at 55 ± 5°C for 24 h and reweighed (dry weight). The brain water content was determined by following equation:

% Brain water content = [(Wet weight – Dry weight)/ wet weight] × 100 and expressed as % brain water content per 100 gm body weight of the animal.

2.8. *Estimation of blood-brain barrier integrity*

For estimation of blood-brain barrier integrity, in the rats of all the groups, Evans blue (2% W/V) was injected intraperitoneally (2 mg/kg, *i.p.*) as per Manaenko *et al*. (*29*) 30 min before craniotomy. All groups except normal were then subjected to craniotomy. The normal as well as craniotomy control group were given vehicle (0.3 % CMC) while the other three groups received single oral administration of agomelatine (1, 3, and 10 mg/ kg). After 24 h, all the animals were anesthetized using thiopentone sodium (40 mg/kg, *i.p.*) and transcardially perfused with ice-cold heparinized saline. The brain was isolated, and Evans blue in each hemisphere was estimated as described in Katayama *et al*. (*30*) with slight modifications. Briefly, each hemisphere was soaked in 0.5 N KOH overnight at 37°C. 2.5 mL of a mixed solution of 4 N H_3PO_4 and acetone (3:15) was added to each tube. The tube was vortexed for a minute and centrifuged at 3,000 rpm for 15 min at 25°C. The absorbance of the blue layer was taken at 620 nm. Evans blue concentration in the brain was calculated using the standard curve prepared from Evans blue.

2.9. *Histopathological study*

The brain tissues were excised, washed with ice-cold 0.9% saline solution and fixed for 24 h in 10% buffered formalin for histopathological studies. The tissues were washed with running tap water to remove any additional fixative. The tissues were finally cleaned with methyl benzoate and embedded in paraffin wax after dehydrating through a graded series of alcohol. Tissue sections of 5 µm thickness were cut, deparaffinized and hydrate to water followed by cresyl violet (0.5% W/V) staining for 3-5 min. After staining tissue sections were dehydrated and fixed by DPX mountant. Sections were examined under a light microscope (200×).

2.10. *Statistical analysis*

Statistical analysis was done using GraphPad Prism 4 (San Diego, California). All the results were expressed as the mean ± SEM ($n = 6$). All the data were statistically analyzed by one-way ANOVA, followed by Newman-Keuls multiple comparisons test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Effect of agomelatine on behavioral performance in craniotomy rats*

Figure 1A illustrates the effect of agomelatine treatment on the score assessed in beam walking in craniotomy induced brain injury model. Results were statistically analyzed using one way ANOVA which indicates significant differences in score among the different groups [$F(4, 29) = 17.23$, $p < 0.0001$]. In craniotomy exposed rats, score decreased significantly compared to the normal control group. A single treatment with agomelatine increased craniotomy induced decreased score at all the doses. Agomelatine in all doses showed significant improvement in performance as compared with craniotomy group.

Figure 1B depicts the effect of agomelatine treatment on the performance of rats on pole test in the craniotomy induced brain injury model. Statistical analysis was done using one way ANOVA [$F(4, 29) = 33.16$, $p < 0.0001$]. A significant difference was found in performance with pole test between normal and craniotomy animals. Agomelatine in a dose of 1, 3, and 10 mg/kg had shown significant improvement in performance compared with

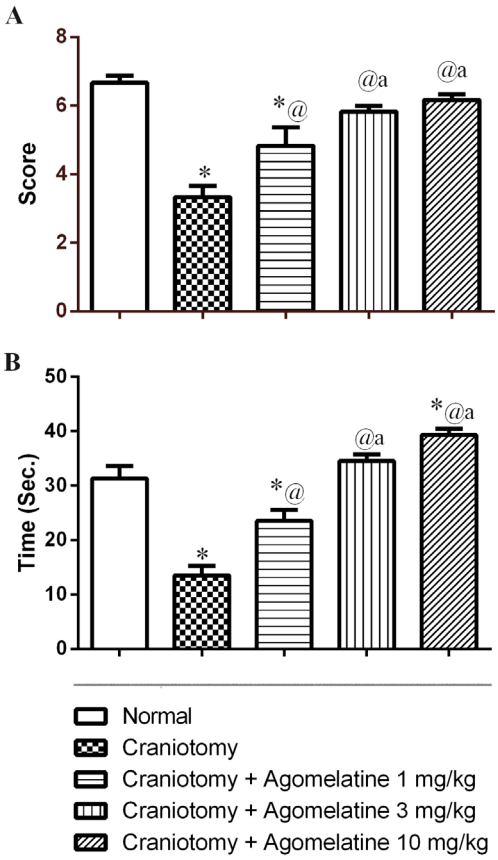

Figure 1. Effect of agomelatine on motor defects induced by craniotomy in rats using beam walking test (A) and pole test (B). Data were expressed as mean ± SEM (n = 6). *p < 0.05 compared to normal, @p < 0.05 compared to craniotomy, [a]p < 0.05 compared to agomelatine 1 mg/kg using one way ANOVA (Newman-Keuls multiple comparison test).

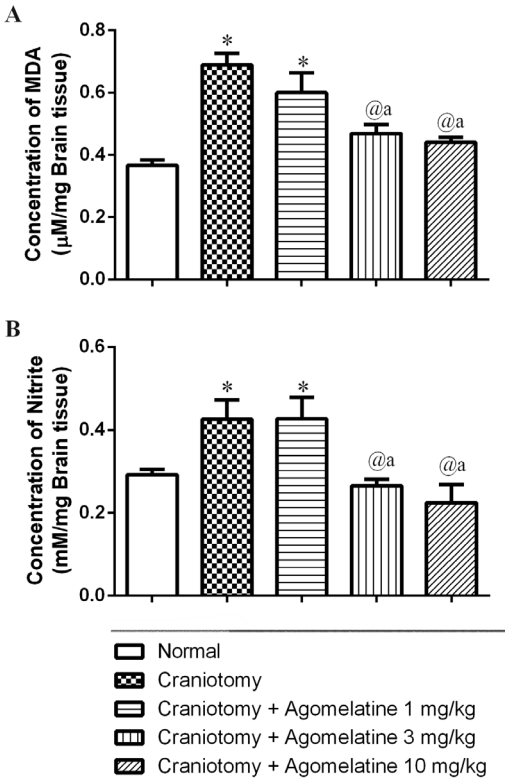

Figure 2. Effect of agomelatine on MDA level (LPO) (A) and nitrite level (B) rats against craniotomy induced brain injury. Data were expressed as mean ± SEM (n = 6). *p < 0.05 compared to normal, @p < 0.05 compared to craniotomy, [a]p < 0.05 compared to agomelatine 1 mg/kg using one way ANOVA (Newman-Keuls multiple comparison test).

the craniotomy group.

3.2. *Effect of agomelatine on oxidative stress and antioxidant parameters*

Figure 2A aptly demonstrates the effect of agomelatine treatment on the level of MDA, a marker of lipid peroxidation. One-way ANOVA revealed significant differences in MDA level among various groups [$F(4,29) = 12.40$; $p < 0.0001$]. Craniotomy did increase MDA level in brain significantly while agomelatine treatment effectively attenuated the craniotomy-induced increase in LPO at the doses of 3 and 10 mg/kg.

Figure 2B represents the effect of agomelatine treatment on nitrite level. One-way ANOVA revealed a significant differences in nitrite level among various groups [$F(4,29) = 6.105$; $p < 0.005$]. It was observed that craniotomy had increased the nitrite level significantly and agomelatine treatment attenuated the increased nitrite level at the doses of 3 and 10 mg/kg.

The results of agomelatine treatment on superoxide dismutase (SOD) levels in craniotomy induced brain injury model were analyzed by one-way ANOVA [$F(4,29) = 7.220$, $p < 0.0005$] (Figure 3A). A significant decrease in SOD levels was observed in craniotomy animals compared to the normal. Agomelatine at all the doses had shown no significant effect on SOD levels compared to the craniotomy group.

The effect of agomelatine treatment on the catalase activity in craniotomy induced brain injury model is shown in Figure 3B. One-way ANOVA revealed a significant differences in catalase activities between groups [$F(4, 29) = 12.01$, $p < 0.0001$]. Post-hoc analysis showed that craniotomy significantly increased catalase activity. Agomelatine (1, 3, and 10 mg/kg, *p.o.*) significantly decreased the catalase activity compared to the craniotomy group.

Figure 3C elucidates the effect of agomelatine treatment on the reduced glutathione in craniotomy induced brain injury. The results were analyzed by one way ANOVA [$F(4,29) = 3.361$, $p < 0.05$]. The significant decrease in reduced glutathione levels was observed in craniotomy animals as compared to normal animals. Agomelatine in the dose of 3 and 10 mg/kg had significantly modulated the reduced glutathione due to craniotomy.

3.3. *Effect of agomelatine on brain water content*

Figure 4 illustrates the effect of agomelatine treatment

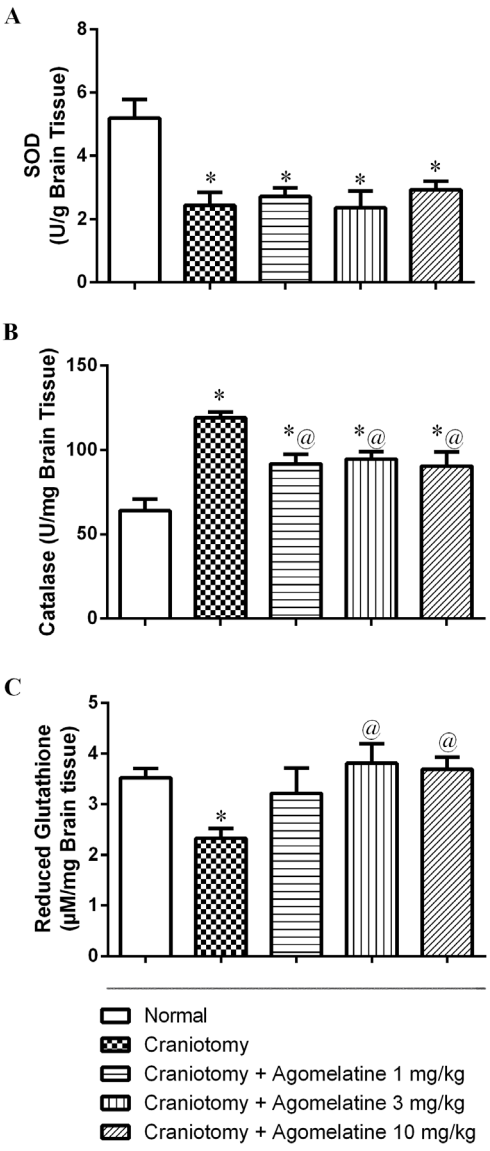

Figure 3. Effect of agomelatine on SOD (A), catalase (B) and reduced glutathione (C) levels in rats against craniotomy induced brain injury. Data were expressed as mean ± SEM ($n = 6$). *$p < 0.05$ compared to normal, @$p < 0.05$ compared to craniotomy, $^a p <$ 0.05 compared to agomelatine 1 mg/kg using one way ANOVA (Newman-Keuls multiple comparison test).

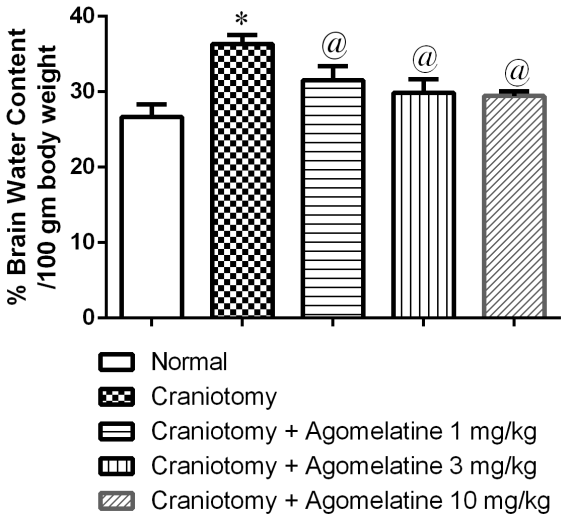

Figure 4. Effect of agomelatine on edema (% brain water content/100 g of body weight) in rats against craniotomy induced brain injury. Data were expressed as mean ± SEM ($n = 6$). *$p < 0.05$ compared to normal, @$p < 0.05$ compared to craniotomy, $^a p < 0.05$ compared to agomelatine 1 mg/kg using one way ANOVA (Newman-Keuls multiple comparison test).

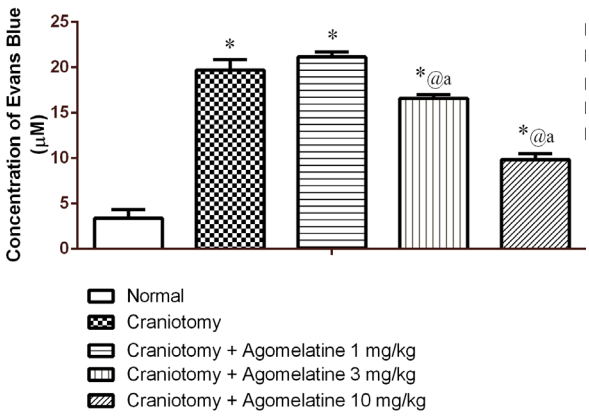

Figure 5. Effect of agomelatine on blood brain barrier (Evans blue concentration) in left and right brain hemisphere of rats against craniotomy induced brain injury. Data were expressed as mean ± SEM ($n = 6$). *$p < 0.05$ compared to normal, @$p < 0.05$ compared to craniotomy, $^a p <$ 0.05 compared to agomelatine 1 mg/kg using one way ANOVA (Newman-Keuls multiple comparison test).

on the water content of brain in animals underwent craniotomy. One-way ANOVA [$F(4,29) = 5.753$, $p < 0.005$] showed that the brain water content increased significantly in the rats undergone craniotomy compared with normal rats without craniotomy. Brain water content was attenuated with the agomelatine treatment at all the doses.

3.4. *Effect of agomelatine on blood-brain barrier integrity*

The effect of agomelatine treatment on the Evans blue concentration in the right brain hemispheres of all the rats was studied and the results were analyzed by one-way ANOVA (Figure 5). Significant differences were observed in the Evans blue concentration among the groups [$F(4, 29 = 89.11$, $p < 0.0001$]. A significant increase in Evans blue concentration was observed in craniotomy animals than normal. Agomelatine in the doses of 3 and 10 mg/kg had shown a significant decrease in Evans blue concentration as compared with craniotomy group.

3.5. *Effect of agomelatine on Nissl staining*

Nissl staining was performed on brain sections from a normal group, craniotomy group, and agomelatine in 1, 3, and 10 mg/kg dose. Sections were stained with cresyl

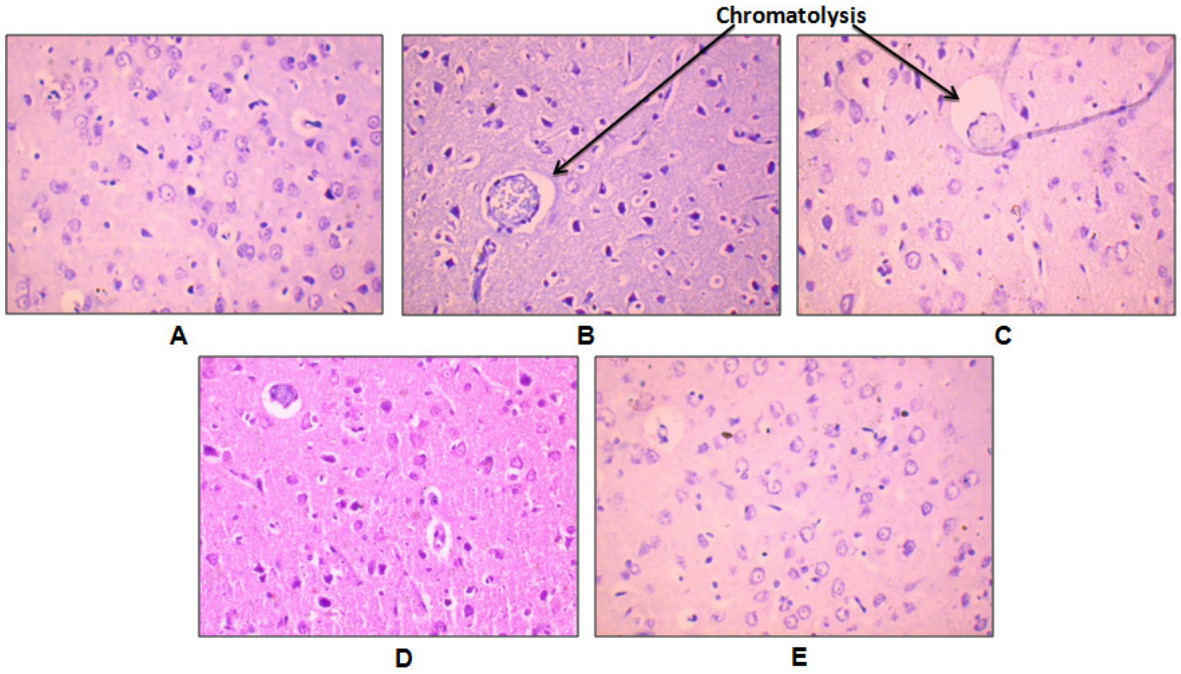

Figure 6. Nissl staining of brain sections of different groups. Normal **(A),** craniotomy **(B),** agomelatine 1 mg/kg **(C),** agomelatine 3 mg/kg **(D),** agomelatine 10 mg/kg **(E).**

violet. Nissl staining using cresyl violet was done in brain sections for detection of neuronal death and damage. Cresyl violet specifically stains the nissl bodies which are large granular bodies in neurons. Chromatolysis (indicated by arrow), *i.e.*, dissolution of nissl bodies was found in craniotomy (Figure 6B) and agomelatine 1 mg/kg groups (Figure 6C). Chromatolysis is an induced response of cell triggered by axotomy, neurotoxicity, cell exhaustion. Chromatolysis is a precursor to apoptosis.

4. Discussion

The key findings of the present investigation are as follows: (1) craniotomy in rats induced increased in brain water content (edema), blood-brain barrier permeability, oxidative stress (LPO and NO), impaired antioxidant mechanisms (SOD, catalase and reduced glutathione) and increased neuronal cell death indicated by augmented chromatolysis, (2) craniotomy also induced impairment in locomotor activity (behavioral consequence) and (3) administration of agomelatine after the craniotomy ameliorated histopathological, neurochemical and behavioral consequences of craniotomy.

Brain edema occurs in TBI. Edema results in increased ICP, which causes death (*4*). The DC is a surgical procedure done to treat increased ICP following TBI. However, DC causes considerable damage to the brain (*6*). The complications after DC are subdural hygroma and hydrocephalus, postoperative hematoma expansion and infection of the surgical site. Consequent cranioplasty has the risk of infection, cerebral edema, and bone flap reabsorption (*31*). Results of present study also shows craniotomy induced brain injury which is evident by increased brain water content (edema), blood-brain barrier permeability, oxidative stress (LPO and NO), impaired antioxidant mechanisms (SOD, catalase, and reduced glutathione) and increased neuronal cell death indicated by augmented chromatolysis visible in nissl stained histopathological slide.

TBI results in neuromotor impairment that causes disabilities affecting mobility, and motor coordination (*32,33*). To elucidate the effect of agomelatine on impairment in mobility, balance and motor coordination induced by craniotomy, we performed two functional tests, the pole test and beam walk test. The craniotomy results in sustained neurological motor dysfunction, as indicated by decreased performance when transverse the beam and the decreased latency time to descend from the top of roughly surfaced pole to the floor. Thus craniotomy induced motor dysfunction. Maier (*34*) noted the cortical area associated with coordinated walking in the rat and a cortical lesion results in a motor abnormality when the rats were transverse a narrow elevated beam. Beam-walking test in rats is an excellent tool for studying the functional recovery of locomotor ability after sensorimotor cortex injury (*35*). Similarly, pole test is a useful method for evaluating the movement disorder (*36*). Hence craniotomy causes the cortical lesions and thus abnormality in mobility and motor coordination as observed with beam walking and pole test. The present study showed that agomelatine in the doses of 1, 3, and 10 mg/kg attenuated craniotomy induced motor deficit in beam walking and pole test. Thus agomelatine increases functional recovery after craniotomy.

Existing evidence suggests that free radicals play a significant role in the pathophysiology of brain injury

(*37*). In the present study, results showed a significant increase in MDA and NO levels in animals underwent the craniotomy. Thus, craniotomy increased overall oxidative stress. Agomelatine attenuated the increase in oxidative stress in craniotomy animals. The brain has many defense mechanisms to protect itself from free radicals. SOD and catalase are antioxidant enzymes, which are involved in the elimination of free radicals, whereas small-molecule antioxidants such as glutathione can repair oxidizing radicals directly (*38*). Results of this investigation show the alteration in anti-oxidants level in craniotomy animals. Agomelatine treatment increased the anti-oxidants level in the brain. Melatonin has been reported to exert direct free radical scavenging activity and indirect antioxidant enzymes stimulatory activity (*39*). Thus, agomelatine normalizes the balance between oxidative stress and anti-oxidants mechanisms impaired by craniotomy *via* its melatonin agonistic activity.

Neural membranes consist of phospholipids in large amount, which are characterized by their high content of some polyunsaturated fatty acids (PUFA) like arachidonic, docosatetraenoic (adrenic), and docosahexaenoic acids. These are essential for normal vascular permeability. Phospholipid peroxidation results in loss of membrane PUFA and contributes to increased membrane rigidity and loss of vascular permeability (*38*). Oxidative-free radicals generated in brain injury are most likely to play a crucial role in increased cerebral vascular permeability and thus in vasogenic brain edema (*40*). The present study showed that the % brain water content/100 g animal body weight was significantly increased in animals underwent craniotomy. This increased brain water content indicates craniotomy induced brain edema. Agomelatine treatment followed by craniotomy attenuated the % brain water content/100 g of body weight of animal thus reduced the brain edema. Blood-brain barrier breakdown and increase of vascular endothelium permeability can result in brain edema and eventually ICP increase, which may be fatal (*41*). The Evans blue is not able to cross the blood-brain barrier. Blood-brain barrier disruption results in increased permeability of Evans blue to the brain. Hence, Evans blue concentration in the brain is indicative of the blood-brain barrier disruption. Craniotomy has shown a marked increase in Evans blue level in the brain. Agomelatine, in a dose of 10 mg/kg, has shown a significant decrease in brain Evans blue concentration. Thus agomelatine mitigated the disturbed blood-brain barrier integrity and increased brain edema caused by craniotomy. MT_1, MT_2, and MT_3 melatonin receptors had anti-edema effects, while MT_1 and MT_2 have a role in protecting the blood-brain barrier (*16*). This beneficial effect of agomelatine on disturbed blood-brain barrier integrity and increased brain edema due to craniotomy might be due to its melatonin agonistic activity.

Free radical generations, reduced antioxidant mechanism, disrupted the blood-brain barrier and increased brain edema results in the neuronal loss. Nissl-staining is a widely used method to study morphology and pathology of neuronal tissue. Histopathological examination of Nissl stained brain sections reveals the chromatolysis in animals underwent craniotomy, which indicates neuronal cell death. The absence of chromatolysis was observed in the nissl stained brain sections of animal treated with agomelatine (3 and 10 mg/kg doses) followed by craniotomy. Thus agomelatine decreased neuronal cell death.

5. **Conclusion**

In the present investigation, the pharmacological evaluation of single treatment agomelatine post craniotomy showed that it possesses promising neuroprotective activity as it mitigates the craniotomy induced histopathological, neurochemical, and behavioral consequences.

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