Research Paper: **Hippocampal Astrocyte Response to Melatonin Following Neural Damage Induction in Rats**

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**Introduction:** Brain injury induces an almost immediate response from glial cells, especially astrocytes. Activation of astrocytes leads to the production of inflammatory cytokines and reactive oxygen species that may result in secondary neuronal damage. Melatonin is an anti-inflammatory and antioxidant agent, and it has been reported to exert neuroprotection through the prevention of neuronal death in several models of central nervous system injury. This study aimed to investigate the effect of melatonin on astrocyte activation induced by Traumatic Brain Injury (TBI) in rat hippocampus and dentate gyrus.

**Methods:** Animals were randomly divided into 5 groups; Sham group, TBI group, vehicle group, and melatonin-treated TBI groups (TBI+Mel5, TBI+Mel20). Immunohistochemical method (GFAP marker) and TUNEL assay were used to evaluate astrocyte reactivity and neuronal death, respectively.

**Results:** The results demonstrated that the astrocyte number was reduced significantly in melatonin-treated groups compared to the vehicle group. Additionally, based on TUNEL results, melatonin administration noticeably reduced the number of apoptotic neurons in the rat hippocampus and dentate gyrus.

**Conclusion:** In general, our findings suggest that melatonin treatment after brain injury reduces astrocyte reactivity as well as neuronal cell apoptosis in rat hippocampus and dentate gyrus.

**Keywords:** Melatonin, Astrocyte, GFAP, Hippocampus

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**ABSTRACT**

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1. Introduction

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entreral nervous system injury induces an almost immediate reaction in glial cells, especially astrocytes (Pekny & Nilsson, 2005; Pineau, Sun, Basliien, & Lacroix, 2010). Astrocytes contribute to brain homeostasis and comprise approximately 90% of total brain mass (Gee & Keller, 2005). Previous studies have shown that primary neuronal injury caused an increase in reactive oxygen species production and inflammatory cytokines released by reactive astrocytes. These conditions may lead to secondary neuronal damage (Asadi-Shekaari, Basin, & Babaee, 2014; Blasko et al., 2004; Ozdemir et al., 2005). The Cornu Ammonis (CA1) region of the hippocampus and neurons in the dentate gyrus (Farina, Aloisi, & Meinl, 2007) are vulnerable to a variety of chemical, physical, and cytotoxic insults (Ansari, Roberts, & Scheff, 2008; Barha, Ishrat, Epp, Galea, & Stein, 2011; Liang et al., 2008). To diminish as much as possible the secondary neurological consequences following injuries of brain tissue, neuroprotective strategies such as using antioxidant compounds are urgently required (Hall, Vaishnav, & Mustafa, 2010).

Melatonin is a tryptophan derivative that is synthesized generally in the pineal gland. This neurohormone controls the circadian rhythm and sleep induction (Hickie & Rogers, 2011; Reiter, Tan, Osuna, & Guito, 2000). Also, melatonin has a better antioxidant and anti-inflammatory effect than vitamin E and can secondarily upsurge the expression of other antioxidant enzymes (Bahaei-Balderlou, Zare, Heydari, & Farokhi, 2010; Boutin, Audinot,erry, & Delargrange, 2005). A recent study has shown that melatonin secretion decreased following neuronal injury (Seifman et al., 2014). Therefore, some researchers, using exogenous administration of melatonin, examined its neuroprotective effects on several models of neural cell injuries (Ding et al., 2014; Dong et al., 2016). For instance, melatonin administration after neonatal hypoxic ischemia reduced neural cell death and reactive astrogliosis (Alonso-Alconada, Alvarez, Alcalde, & Hilaro, 2012). Brain injury research until quite recently has focused on the pathophysiology of injured neurons, while very little attention has been paid to non-neuronal cells (Barreto, Gonzalez, Torres, & Morales, 2011; Burda, Bernstein, & Sofroniew, 2016).

Because of inadequate attention to the melatonin effects on astrocytes reactivity and vulnerability of nervous tissue to inflammation and oxidative stresses (Leszek, Barreto, Gasiorowski, Koutsouraki, & Aliev, 2016), we investigate the effects of exogenous administration of melatonin on astrocyte activation induced by traumatic brain injury in rat hippocampus and dentate gyrus.

2. Methods

2.1. Animals and experimental protocols

All experiments were done in accordance with the rules of the Ethics Committee of Medical Faculty (EC/ KNRC/90-2; Kerman University, Iran). A total of 35 male rats (NMRI, 230 to 275 g) were kept in the animal cage with free access to food and water. Animals were randomly divided into 5 groups before Traumatic Brain Injury (TBI) induction: Sham (intact) (n=7), TBI group (n=7), vehicle (n=7) that exposed to TBI and received an intraperitoneal injection of melatonin vehicle (ethanol + normal saline; 0.33 mL/rat) (Chern, Liao, Wang, & Shen, 2012; Dehghan, Hadad, Asadikram, Najafipour, & Shahrokhi 2013), and two TBI+melatonin groups that...
were exposed to TBI and then received 5 mg/kg and 20 mg/kg melatonin (Sigma, St. Louis, MO), respectively at 1 hour, and then 1, 2 and 3 days post-TBI (Babaee et al., 2015; Gutierrez-Cuesta et al., 2007).

2.2. Induction of TBI

After incision in the skull skin, the animals were exposed to diffuse brain trauma through the Marmarou method (Marmarou et al., 1994). In this method, the skull of animals was enclosed with a metallic disc (with 3 mm thick and a diameter of 10 mm), and then a 250 g weight (at the height of 2 m) was thrown onto the skull of the rats. Following recovery, the rats were returned to their cages (Babaee et al., 2015; Marmarou et al., 1994).

2.3. Preparing the brain tissue

For histological evaluation, all rats were anesthetized with IP injection of 400 mg/kg chloral hydrate (Merck, 102425) (Mortezazadeh et al., 2018) 72 h after brain trauma and were perfused intracardially with 140-180 mL of heparinized 0.9% saline, followed by 100 to 120 mL of 4% paraformaldehyde in Phosphate-Buffered Saline (PBS). The perfusion continued until the lungs and liver were clear of blood (for 10 to 15 min) (Babaee et al., 2019; Pourhoseini et al., 2017). Then, animals’ brains were cautiously removed and immediately transferred in 10% formaldehyde and maintained overnight at 4°C. The brains were then dehydrated by alcohol solution and finally embedded in paraffin (Babaee et al., 2015; Ding et al., 2014).

2.4. TUNEL staining

TUNEL staining was carried out on the cerebral sections using the apoptosis detection kit (Indianapolis, Roche, IN). Staining of sections was performed according to the manufacturer’s instructions. Afterward, cerebral sections were incubated with DAB (3, 3’-diaminobenzidine) and hydrogen peroxide; the brownish color shows damage to neuronal perikarya (Hakemi, Sharififar, Haghpanah, Babaee, & Eftekhar-Vaghefi, 2019; Varshosaz, Taymouri, Pardakhty, Asadi-Shekaari, & Babaee, 2014). Also, negative and positive controls were included.

2.5. Immunohistochemistry

For assessing astrocytic reactivity, we used mouse monoclonal anti-GFAP (Giall Fibrillary Acidic Protein) antibody (Dako A/S Denmark, 1:400) (Dehghani-Soltani, Shojaee, Jalalkamali, Babaee, & Nematollahi-Mahani, 2017). For this purpose, after deparaffinization in a microwave oven with 65°C and rinsing of sections in xylene (5 min), they were washed with PBS and were put in the 10 mM citrate buffer for 1 h (temperature of 90°C and pH 6). Then the sections were placed in 0.3% H2O2 for 12 minutes. After overnight incubation with primary antibody, the sections were washed with PBS and then incubated with secondary antibody (1:350, rabbit anti-mouse) for 2 hours at room temperature (Babaee et al., 2015; Babaee et al., 2018).

2.6. Cell counting

Five coronal sections of the hippocampus were chosen for cell counting. In each section, the TUNEL positive cells were counted in four fields of dentate gyrus and hippocampus at 200× magnification (Pazar et al., 2016). Additionally, GFAP positive cells (astrocytes) were counted in four random and non-overlapping regions (Eftekhar-Vaghefi, Raygan, Eftekhar-Vaghefi, Dehghani-Soltani, & Babaee, 2017; Soltani et al., 2016) of the hippocampus and dentate gyrus using an optical microscope (TS100, Nikon, Japan).

2.7. Statistical analysis

The obtained data were expressed as Mean±SEM. One-way ANOVA followed by Tukey-Kramer multiple post hoc test was used to assess the significant differences (P<0.05) between groups (Mortazavi, 2009).

3. Results

After preparation of coronal sections, including the hippocampus and dentate gyrus, some of them were stained with hematoxylin and eosin for morphological evaluation (Figure 1), and the others were subjected to TUNEL staining and immunohistochemistry.

Figure 1. A coronal section of the rat brain that indicated the cornu Ammonis regions (CA1, CA2, CA3) of the hippocampus as well as Dentate Gyrus (DG) (H&E staining, 100×).
3.1. Evaluation of apoptotic cells

Our findings showed that apoptotic cells were significantly increased (P<0.001) in dentate gyri and hippocampi of TBI group rats in comparison to the sham group, while the number of apoptotic neurons in the melatonin-treated groups was significantly decreased (Figures 2 and 3) that were not dose-dependent.

3.2. The number of activated astrocytes

In the present study, increased GFAP-positive astrocytes were detected in the hippocampi and dentate gyri of TBI group rats compared to that in the sham rats (Figures 4 and 5). Also, treatment with melatonin significantly (P<0.01) reduced activated astrocytes that were not dose-dependent (Figure 6).

4. Discussion

The results of the current study have demonstrated the neuroprotective effect of melatonin after traumatic injury in rats. Acquired data indicate that TBI leads to a significant increase in neuronal cell death in the hippocampus and dentate gyri, and based on TUNEL staining, the number of apoptotic cells noticeably decreased in the melatonin treatment groups (Mel5 and Mel20). Gao et al. reported that traumatic brain injury causes synaptic and dendritic degeneration in the dentate gyrus (Gao, Deng, Xu, & Chen, 2011). Also, Hung et al. have shown that melatonin alleviates hippocampal injury following hypoxia (Hung, Tipoe, Poon, Reiter, & Fung, 2008). Annually, more than 1 million people die following TBI worldwide (Babaee et al., 2015). Neural inflammation and production of oxidative stress are two major pathological mechanisms of neuronal cell death after TBI (Cornelius et al., 2013; Woodcock & Morganti-Koss...
To inhibit secondary injuries after primary brain injury, it is essential to limit the neural inflammation via decreasing the astrocyte activation (Kabadi, Stoica, Loane, Luo, & Faden, 2014). Chern et al. have shown that intraperitoneal administration of melatonin improved the neuronal survival rate in ischemic-stroke mice (Chern et al., 2012).

Besides, melatonin has been assessed as an effective medicine in TBI through increasing glutathione peroxidase and superoxide dismutase activities (Dehghan et al., 2013). The result of this study does not show any noticeable difference between two different doses of melatonin. Ozdemir et al. reported that melatonin significantly reduced oxidative damage induced by TBI in immature rats, which was equally effective at different doses of 5 mg/kg and 20 mg/kg (Ozdemir et al., 2005).

Astrocytes, as the most abundant glial cells in brain tissue (Farina et al., 2007), maybe the target of melatonin. Our findings have shown that the number of astrocytes is decreased in the melatonin treatment groups, which shows the alleviation of astrogliosis induced by TBI. In response to different injuries of brain tissue, such as trauma, chemical injuries, tumor formation, brain ischemia, and neurodegenerative disease (Guo et al., 2014; Hald, Nedergaard, Hansen, Ding, & Heegaard, 2009; Lee et al., 2010). Activation of astrocytes following brain injury has known as astrogliosis. Previous studies have demonstrated that up-regulation of GFAP occurs during the astrogliosis phenomenon (Hostenbach, Cambrun, D’haeseleer, Kooijman, & De Keyser, 2014; Kamphuis et al., 2012). Therefore, this study has focused on astrocyte activation based on GFAP immunoreactivity.

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Glial cells activation, particularly astrocytes, occurs in response to different injuries of brain tissue, such as trauma, chemical injuries, tumor formation, brain ischemia, and neurodegenerative disease (Guo et al., 2014; Hald, Nedergaard, Hansen, Ding, & Heegaard, 2009; Lee et al., 2010). Activation of astrocytes following brain injury has known as astrogliosis. Previous studies have demonstrated that up-regulation of GFAP occurs during the astrogliosis phenomenon (Hostenbach, Cambrun, D’haeseleer, Kooijman, & De Keyser, 2014; Kamphuis et al., 2012). Therefore, this study has focused on astrocyte activation based on GFAP immunoreactivity.

Figure 4. Immunohistochemical analysis of GFAP-positive cells
Hippocampal astrocytes in Sham group (A); TBI group; and (B); TBI+Mel20 group; Arrows display the astrocytes (Scale bar=35 µm).

Figure 5. Immunohistochemical analysis of GFAP-positive cells in the dentate gyrus of different groups
A: Sham group; B: TBI group; and TBI + Mel20 group (Scale bar=15 µm).
an experimental study, Ananth et al. demonstrated that domoic acid-induced astrogliosis is attenuated significantly in the hippocampus of adult rats using exogenous administration of melatonin (Ananth, Gopalakrishnake, & Kaur, 2003). Barreto et al. reported that astrocytic-neuronal interactions could act as a neuroprotective strategy against brain injury (Barreto et al., 2011).

The blood brain barrier has an important role in exacerbating neuronal damage following traumatic brain injury (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; Persidsky, Ramirez, Haorah, & Kanmogne, 2006). In other words, damage of this barrier resulted in entrance of neutrophils, lymphocytes and monocytes to the injured site. Glial cells are then activated and induce inflammation that may promote neuronal death (Seo et al., 2013; Ziebell & Morganti-Kossmann, 2010).

In addition, Tsai et al. demonstrated that melatonin administration reduced proinflammatory cytokines via up-regulation of STAT1 DNA binding activity (Tsai, Chen, Tsai, Ching, & Chuang, 2011). However, the modulation actions of melatonin to astrocyte, which was introduced as the primary source of proinflammatory cytokines such as IL-6β, have not yet been widely examined. Our experiment only shows the neuroprotective effects of melatonin following a short period after brain injury (1 h to 72 h). Thus, additional investigations need to be done to elucidate the molecular mechanism of melatonin to alleviate astrocyte reactivity.

Ethical Considerations

Compliance with ethical guidelines

Animal procedures in this research were done in compliance with the Guide for the Care and Use of Laboratory Animals and certified by the local Animal Ethics Committee of Kerman University of Medical Sciences (Kerman, Iran) in 2016 (EC/KNRC/90-2).

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Authors’ contributions

Conceptualization, data collection, and writing original draft: Abdolreza Babaee, Samereh Dehghani-Soltani; Data analysis, writing – review & editing, investigation, supervision: Seyed Hassan Eftekhar-Vaghefi Majid Asadi-Shekaari, Nader Shahrokhi, Mohsen Basiri.

Conflict of interest

The authors declared no conflict of interest.

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