Title: Hippocampal Astrocyte Response to Melatonin Following Neural Damage Induction in Rat

Running title: Hippocampal Astrocyte Response to Melatonin

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To appear in: Basic and Clinical Neuroscience

Received date: 2016/08/28
Revised date: 2019/11/8
Accepted date: 2019/05/18

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Please cite this article as:


DOI: http://dx.doi.org/10.32598/bcn.9.10.415
Abstract

Introduction: Brain injury induces an almost immediate response from glial cells, especially astrocytes. In fact, activation of astrocytes leads to 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 prevention of neuronal death in several models of central nervous system injury. In this study, we 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 was used to evaluate astrocyte reactivity and neuronal death, respectively.

Results: The results demonstrated that, the number of astrocyte 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 rat hippocampus and dentate gyrus.

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

Keyword: Melatonin, Astrocyte, GFAP, Hippocampus
Introduction:

Central nervous system (CNS) injury induces an almost immediate reaction from glial cells especially astrocytes (Pekny & Nilsson, 2005; Pineau, Sun, Bastien, & 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 as well as inflammatory cytokines release by reactive astrocytes. These conditions may lead to secondary neuronal damage (Asadi-Shekaari, Basiri, & Babaei, 2014; Blasko et al., 2004; Ozdemir, Uysal, Gonenc, & Acikgoz, 2005). The cornu ammonis (CA1) region of hippocampus and neurons in the dentate gyrus (Farina, Aloisi, & Meinl) are vulnerable to a variety of chemical, physical and cytotoxic insults (Ansari, Roberts, & Scheff, 2008; Barha, Ishrat, Epp, Gales, & 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, & Gitto, 2000). Also melatonin has a better Antioxidant and anti-inflammatory effect than vitamin E, and can secondarily upsurge the expression of other antioxidant enzymes (Babaei-Balderlou, Zare, Heidari, & Farrokhi, 2010; Boutin, Audinot, Ferry, & Delagrange, 2005). A recent study has shown that melatonin secretion decreased following neuronal injury (Seifman et al., 2014). Therefore, some researchers by means of exogenous administration of melatonin examined its neuroprotective effects on several models of neural cell injuries (Ding et al., 2014; Dong et al., 2015). For instance, melatonin administration after neonatal hypoxic ischemia reduced neural cell death and reactive astrogliosis (Alonso-Alconada, Alvarez, Lacalle, & Hilario, 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 not adequate attention has been paid to the melatonin effects on astrocytes reactivity and since nervous tissue is vulnerable to inflammation and oxidative stresses (Leszek et al., 2016), we investigate effects of exogenous administration of melatonin on astrocyte activation induced by traumatic brain injury in rat hippocampus and dentate gyrus.
Materials and methods:

Animals and experimental protocols
All experiments were done in accordance with the rules of Ethics Committee of Medical faculty (EC/KNRC/90-2; Kerman University, Iran). A total of 35 male rats (NMRI, 230 to 275g) were kept in the animal room with free access to food and water. Animals were randomly divided into 5 groups before traumatic brain injury (TBI) induction; Sham (intact) group (n=7); TBI group (n=7); vehicle group (n=7) that exposed to TBI and received an intraperitoneally injection of melatonin vehicle (ethanol + normal saline; 0.33 ml/rat) (Chern, Liao, Wang, & Shen, 2012; Dehghan, Hadad, Asadikram, Najafipour, & Shahrokhi, 2013), and 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, 1, 2 and 3 days post-TBI (Babaee et al., 2015; Gutierrez-Cuesta et al., 2007).

Induction of TBI
After incision in the skull skin, the animals 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 (whit 3 mm thick and a diameter of 10 mm) and then a 250 g weight (from a height of 2 meters), was thrown onto the skull of the rats and following recovery, they were returned to their cages (Babaee et al., 2015; Marmarou et al., 1994).

Preparing the brain tissue
For histological evaluation, all rats were anesthetized with i.p. injection of 400 mg/kg chloral hydrate (Merck, 102425) (Mortezaazadeh et al., 2018) at 72 h after brain trauma, and 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). Indeed perfusion continued until the lungs and liver were clear of blood (for 10 to 15 min)(Babaee, Nematollahi-Mahani, Dehghani-Soltani, Shojaei, & Ezzatabadipour, 2019; Pourhoseini et al., 2017). Then, the brain of animals 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).

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 incubation of cerebral sections with DAB (3, 3’-diaminobenzidine) as well as hydrogen peroxide, brownish color shown damage to neuronal perikarya (Hakemi, Sharififar, Haghipanah, Babaee, & Eftekhar-Vaghefi, 2019; Varshosaz, Taymouri, Pardakhty, Asadi-Shekaari, & Babaee, 2014). Also negative and positive controls were included.

**Immunohistochemistry**

In this study for assessment of astrocytic reactivity we used Mouse monoclonal anti-GFAP antibody (Dako A/S Denmark, 1:400) (Dehghani-Soltani, Shojaei, 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 washed with PBS and were put in the 10 mM citrate buffer for 1 h (temperature of 90°C and pH of 6). Then the sections was placed in H2O2 (0.3%) for 12 minutes. After overnight incubation with primary antibody, the sections washed with PBS and then were incubated with secondary antibody (1:350, rabbit anti-mouse) for 2 hours at room temperature (Babaee et al., 2015; Babaee, Nematollahi-Mahani, Shojaei, Dehghani-Soltani, & Ezzatabadipour, 2018).

**Cell counting**

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

**Statistical analysis**

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 (Seyed Mohammad Javad Mortazavi, 2009).

**Results**

After preparation of coronal sections including 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.
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 sham group, while the number of apoptotic neurons in the melatonin-treated groups was significantly decreased (Figure 2 and 3) that were not dose dependent.

![Figure 2: Neuronal apoptosis; Control group (A), arrows show the healthy neurons. TBI group (B), arrows show the TUNEL positive cells (apoptotic neurons). Scale bar = 15 µm.](image)

![Figure 3: Melatonin treatment reduced apoptosis that induced by TBI in hippocampi (A) and dentate gyri (B) of rats. Results are expressed as mean±SEM. (** differences Vs sham group; ++ differences Vs TBI group).](image)

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 (Figure 4 and 5). Also,
treatment with melatonin significantly ($P<0.01$) reduced activated astrocytes that were not dose dependent (Figure 6).

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

Figure 5: Immunohistochemical analysis of GFAP positive cells in dentate gyrus of different groups. Sham group (A), TBI group (B) and TBI + Mel20 group (Scale bar = 15 µm).

Figure 6: Melatonin treatment decreased astrocytes in hippocampi (A) and dentate gyri (B) of rats following TBI induction. Results are presented as mean±SEM (*** differences Vs sham group; ++ differences Vs TBI group).
Discussion

The results of current study have demonstrated the neuroprotective effect of melatonin after traumatic injury in rat. Acquired data indicate that TBI lead to a significantly increase in neuronal cell death in hippocampus and dentate gyrus, and based on TUNEL assay, 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 gurus (Gao, Deng, Xu, & Chen, 2011). Also Hung et al. have shown that melatonin alleviate hippocampal injury following hypoxia (Hung, Tipoe, Poon, Reiter, & Fung, 2008).

Annually, more than 1 million people die following TBI worldwide (Babae 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-Kossmann, 2015). In order to inhibition of secondary injuries due to primary brain injury, it is essential to limit the neural inflammation via decrease the astrocyte activation (Kabadi, Stoica, Loane, Luo, & Faden, 2014). Chern et al. have shown that intraperitoneally administration of melatonin improved the neuronal survival rate in ischemic-stroke mice (Chern et al., 2012).

In addition, 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).

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 astrogliosis phenomenon (Hostenbach, Cambron, D’haeseleer, Kooijman, & De Keyser, 2014; Kamphuis et al., 2012). Therefore, this study has focused on astrocyte activation based on GFAP immunoreactivity.

Indeed, astrocytes, as the most abundant glial cells in brain tissue (Farina et al., 2007), may be the target of melatonin. Our findings have shown that the number of astrocyte is decreased in the melatonin treatment groups, which shows the alleviation of astrogliosis induced by
TBI. In 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, Gopalakrishnakone, & Kaur, 2003). Barreto et al. reported that astrocytic-neuronal interactions can act as a neuroprotective strategy against brain injury (Barreto et al., 2011).

Also, previous reports have shown that the blood brain barrier has a key role in pathological changes during acute post-traumatic period (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; Persidsky, Ramirez, Haorah, & Kanmogne, 2006). Destruction of BBB following brain injury allows the entry of neutrophils, lymphocytes and monocytes into the injured site, which affects glial cell reactivities and inflammation that may cause neural cell death (Seo et al., 2013; Ziebell & Morganti-Kossmann, 2010).

In addition, Tsai and colleagues demonstrated that melatonin administration led to the reduction of 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 introduced as a main 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 time period after brain injury (1h to 72h). Thus, additional investigations need to be done to elucidate the molecular mechanism of melatonin in alleviation of astrocyte reactivity.

References


treatment reduces astrogliosis and apoptosis in rats with traumatic brain injury. *Iranian journal of basic medical sciences, 18*(9), 867.


Liang, Hua-Wei, Qiu, Shui-Feng, Shen, Jia, Sun, Li-Na, Wang, Jing-Ye, Bruce, Iain C, & Xia, Qiang. (2008). Genistein attenuates oxidative stress and neuronal damage following transient global cerebral ischemia in rat hippocampus. *Neuroscience letters, 438*(1), 116-120.


