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Comparison of therapeutic effects of melatonin by two different routes in focal cerebral ischemic rats

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ABSTRACT
Objective: To explore the therapeutic effects of melatonin by two different routes, by caudal vein and intraperitoneal injection, on cerebral ischemia-reperfusion (IR) injury in adult rats.

Methods: 60 Sprague-Dawley rats were randomly divided into normal control (CON), middle cerebral artery occlusion (MCAO), intraperitoneal and caudal vein injection groups. Nissl and immunohistochemical staining were used to observe the morphological and quantitative changes of neurons and the expression of cleaved Caspase-3, Fas and FasL proteins in the injured cerebral cortex.

Results: More Nissl-stained and NeuN+ cells were observed in both the intraperitoneal and caudal vein injection groups as compared with the MCAO group (P < 0.05), and the number of Nissl-stained and NeuN+ cells in caudal vein injection group was significantly higher than in intraperitoneal injection group at each time point (all P < 0.05). There were fewer cleaved Caspase-3+, Fas+ and FasL+ cells in both intraperitoneal and caudal vein injection groups than that in MCAO group 24 hours and 72 hours after IR (all P < 0.05). Meanwhile, there were significantly fewer cleaved Caspase-3+, Fas+ and FasL+ cells in caudal vein injection group than in intraperitoneal group (all P < 0.05).

Conclusions: Melatonin therapy by both intraperitoneal and caudal vein injection could alleviate the expression of cleaved Caspase-3, Fas and FasL proteins in the cerebral cortex in rats after cerebral ischemia reperfusion and protect the neurons from injury, and had neuroprotective effects, and the therapeutic effect by caudal vein injection was better than by intraperitoneal injection.

1 Introduction

Ischemia-reperfusion (IR) injury is regarded as the dysfunction of tissue or organ induced by ischemia and which cannot be resumed after the blood supply is restored by treatment [1]. Cerebral IR can lead to severe neurological damage, which is mainly caused by mitochondrial injury, oxygen free radical damage, inflammation, calcium overload, and apoptosis of brain cells [2]. Pharmaceutical drug plays an important role in treatment of cerebral ischemia-reperfusion injury. Melatonin (MT), a kind of indole-like hormone secreted by pineal gland, has high liposolubility and free passage through cell membrane and blood-brain barrier. MT plays an important role in the treatment of nerve injury and it is characterized by antioxidant and anti-inflammation activity, therefore it attracts people's great interest in the research of cerebral ischemia-reperfusion injury [3]. Although many studies have described the antioxidant and anti-inflammation characteristics of MT therapy for IR injury, and our previous study showed that MT could alleviate white
matter injury in cerebral IR rats [4], the therapeutic effect of MT by different routes on the grey matter injury in cerebral IR rats remained unclear. And it is of great importance to select the right MT therapeutic routes for the cerebral IR injury. Here we investigated the effect of MT, by both caudal vein and intraperitoneal injection, on the gray matter damage and apoptosis of cerebral cortex neurons in rats with cerebral ischemia reperfusion.

2 Materials and methods

2.1 Experimental animals

A total of 60 healthy male adult Sprague-Dawley rats, with a body mass of 250~300 mg, were purchased from the Jinan Pengyue Experimental Animal Breeding Co., Ltd..

2.2 Animal groups

Rats were randomly divided into control group (CON group, \( n = 6 \)), middle cerebral artery occlusion group (MCAO group, \( n = 18 \)), intraperitoneal injection group (\( n = 18 \)) and caudal vein injection group (\( n = 18 \)). MT (5 mg/kg) was injected respectively through intraperitoneal and caudal vein before and after modeling in the intraperitoneal and caudal injection groups. In the CON group, the carotid artery was only separated with no operation.

2.3 Main reagents and instruments

Melatonin (Sigma-Aldrich Company); Fas, FasL (Wuhan Boster Biological Technology, Ltd.); Rabbit Antibody to cleaved Caspase-3 (Cell Signaling Technology Company, USA); NeuN antibody (Wuhan Boster Biological Technology, Ltd.); Toluidine blue dye (Beijing Solarbio Biotechnology Co., Ltd.); Positive optical microscope (Olympus, Japan); Electrothermal thermostat (Binder, Germany).

2.4 MCAO model

The MCAO model was made according to the modified Longa thread occlusion method [5] and was evaluated by Longa method to determine whether it was successful or not.

2.5 Extraction

The rats in each group were performed intraperitoneally anesthesia after IR 24 hours, 72 hours and 7 days, their brains were then extracted after cardiac perfusion with normal saline and 4% paraformaldehyde. After being fixed with 4% paraformaldehyde for 24 hours, the brains were dehydrated and embedded in paraffin, and the coronal sections were cut into 4 \( \mu \)m thickness slices.

2.6 Nissl staining

After being dewaxed and hydrated, the tissues on the slices were stained with Toluidine blue. Then these slices were incubated at 37 °C for 30 minutes. Then slices were, in turn, rinsed with distilled water, decolorized with 95% alcohol, dehydrated with anhydrous ethanol, clarified with xylene, and finally sealed with neutral gum.

2.7 Immunohistochemical staining

Brain specimens were fixed with 10% paraformaldehyde, embedded in paraffin, cut into 4 \( \mu \)m sections, deparaffinized with xylene and rehydrated by decreasing concentrations of ethanol. Antigen retrieval was performed for 10 min at 98 °C in citrate buffer. Sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidases, incubated at 37 °C for 1 hour with antibodies to Caspase-3, Fas and FasL(all dilution 1:100), then were incubated overnight at 4 °C, followed by incubation with goat anti-rabbit IgG polymer. Sections were visualized using DAB chromogen substrate and were counterstained with haematoxylin.

2.8 Cell counting

Five visual fields were randomly selected in the ischemic cerebral cortex from each slice and 5 discontinuous slices were selected from each rat at different time points. All the images were analyzed and the cleaved Caspase-3, Fas, FasL and NeuN positive cells were calculated using CellSens Dimension 1.6 software.

2.9 Statistical analysis

One-way ANOVA with SNK-Q was used to test differences between groups. \( P < 0.05 \) was considered
significant. Statistical analyses were performed in SPSS version 22.0.

3 Results

3.1 Effects of MT therapy by different pathways in the lesioned cerebral cortex neurons

Nissl body, the characteristic structure of mature neurons, can reveal the stage of neurons. In CON group, the cerebral cortex neurons with abundant Nissl bodies were arranged regularly and densely, compared with irregular cellular morphology in MCAO group. The number of Nissl-stained cells in the cerebral cortex decreased from 24 hours after IR in MCAO group, intraperitoneal injection group and caudal vein injection group. Compared with CON group, there were fewer Nissl-stained cells in all three groups at each time point after IR ($P < 0.05$). Nissl-stained cells in intraperitoneal group and caudal vein group were more than MCAO group at 24 hours, 72 hours and 7 days after IR ($P < 0.05$), and a higher level of Nissl-stained cells in caudal vein group than in intraperitoneal group was observed ($P < 0.05$, Fig. 1).

3.2 Effects of MT therapy by different pathways on Caspase-3 expression in lesioned cerebral cortex

Caspases are crucial mediators of cell apoptosis and Caspase-3 is the executor. The expression of cleaved Caspase-3 indicates the occurrence of apoptosis. We detected cleaved Caspase-3 positive cells by immunohistochemical staining. The results showed that in CON group, the cerebral cortex cells were arranged neatly and cleaved Caspase-3 positive cells were rarely found. While in MCAO group, the number of cleaved Caspase-3 positive cells began to rise 24 hours after IR, and reached the peak at 72 hours. There was significant difference between CON group and MCAO group at each time point after IR (all $P < 0.01$). At 24 hours and 72 hours after IR, fewer cleaved Caspase-3 positive cells in both intraperitoneal and caudal vein injection group were observed compared with MCAO group, but still more than CON group (all $P < 0.05$). Also, there was a statistical difference between intraperitoneal group and caudal vein group at 24 hours and 72 hours after IR (both $P < 0.05$). On 7 days after IR, there was no group difference in the number of cleaved Caspase-3 positive cells between MCAO group and intraperitoneal group ($P > 0.05$), but compared with both MCAO group and intraperitoneal group, cleaved Caspase-3 positive cells in caudal vein group were less, and the differences were statistically significant (all $P < 0.05$, Fig. 2).

3.3 Effects of MT therapy by different pathways on Fas expression in lesioned cerebral cortex

Fas protein induces the process of neuronal apoptosis after brain injury, and the expression of Fas protein in the brain is related to the degree of apoptosis. We used

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Fig. 1 Comparison of MT therapeutic effects by different routes on neurons in lesioned cerebral cortex. (A–D) show the results of Nissl-stained cells in lesioned cerebral cortex 72 hours after IR (scale bar = 20 μm). (A) CON group, there existed clear and abundant Nissl-stained cells. (B) MCAO group, there existed fuzzy Nissl-stained cells. (C) Intraperitoneal injection group, Nissl-stained cells were obviously observed. (D) Caudal vein injection group, Nissl-stained cells were obviously observed. (E) The changes of the number of Nissl-stained cells in cerebral cortex neurons at different time points after IR. *, $P < 0.01$ compared with CON group; #, $P < 0.05$ compared with MCAO group; **, $P < 0.05$ compared with intraperitoneal injection group.
immunohistochemical staining to observe Fas\(^+\) cells, which showed that rare Fas expression in CON group. Whereas, in MCAO group, the number of Fas\(^+\) cells was significantly higher than CON group with a raise at 24 hours after IR. At 24 hours and 72 hours after IR, in intraperitoneal group and caudal vein group, the number of Fas\(^+\) cells was significantly decreased compared with MCAO group (all \(P < 0.05\)). Moreover, there was a significant difference between intraperitoneal group and caudal vein group at 24 hour and 72 hour after IR (both \(P < 0.05\)). On 7 days after IR, there was no statistical difference between MCAO group and intraperitoneal group on the number of Fas\(^+\) cells (\(P > 0.05\)). However, compared with both MCAO group and intraperitoneal group, the level of Fas\(^+\) cells in the caudal vein group was lower, and the differences were statistically significant (all \(P < 0.05\), Fig. 3).

3.4 Effects of MT therapy by different pathways on FasL expression in lesioned cerebral cortex

FasL, the ligand of Fas, combines with Fas to induce neuronal apoptosis. FasL\(^+\) cells appear to be stained yellow brown by DAB in immunohistochemical staining. The results showed that there were rarely FasL\(^+\) cells in CON group. Whereas, in MCAO group the number of FasL\(^+\) cells increased at 24 hours and reached the peak at 72 hours after IR, which was significantly higher than CON group. At 24 hours and 72 hours after IR, in intraperitoneal group and caudal vein group, the number of FasL\(^+\) cells decreased compared with MCAO group (all \(P < 0.05\)). Moreover, there was a significant difference between intraperitoneal group and caudal vein group at 24 hour and 72 hour after IR (both \(P < 0.05\)). On 7 days after IR, there was no statistical difference between MCAO group and intraperitoneal group on the number of FasL\(^+\) cells (\(P > 0.05\)). However, compared with both MCAO group and intraperitoneal group, the level of FasL\(^+\) cells in caudal vein group was lower, and the differences were statistically significant (all \(P < 0.05\), Fig. 4).

3.5 Effects of MT therapy by different pathways on NeuN\(^+\) expression in lesioned cerebral cortex.

Neuronal nuclei (NeuN) is a mature neuron marker, and NeuN\(^+\) cells present brown granules in the nucleus. The results showed that there were a large number of NeuN\(^+\) neurons in cerebral cortex in CON group. The number of NeuN\(^+\) cells in cerebral cortex was significantly decreased in MCAO group compared with CON group at each time point after IR, which was statistically different (\(P < 0.05\)). At 24 hours, 72 hours and 7 days after IR, the number of NeuN\(^+\) cells in intraperitoneal group and caudal vein group was statistically higher compared with MCAO group, but
still lower than CON group ($P < 0.05$). There was significant difference between caudal vein group and intraperitoneal group on the number of NeuN$^+$ cells at each time point after IR ($P < 0.05$, Fig. 5).

4 Discussion

Cerebral ischemia-reperfusion injury is induced by the interruption of cerebral blood supply and the restoration of cerebral blood supply [6]. This study investigated the therapeutic effects of MT by different injection methods in rats after cerebral IR. The animal models showed physical activity restrictions, focal ischemic lesions in the brain and neuron apoptosis in the ischemic side of the cerebral cortex. The damage in the brain and neuronal apoptosis were alleviated after MT therapy via intraperitoneal and casual vein injection.
Neuronal nuclei (NeuN), a specific marker of mature neurons, mostly distributes in cytoplasm and nucleus [7]. Under pathological conditions such as ischemia, hypoxia and nerve injury, the NeuN immune response decreased due to neuronal apoptosis [8]. We had observed the NeuN immunological activity of cortical neurons in four groups, which showed that the level of NeuN positive cells in MCAO group was the lowest. In intraperitoneal injection group and caudal vein injection group the level was higher than MCAO group, but still less than CON group. Moreover, the NeuN positive cells in caudal vein group were more than that in intraperitoneal group. All the results suggested that MT could reduce nerve cell death and cerebral ischemia-reperfusion injury, and the effect of caudal vein injection was better than that of intraperitoneal injection. This may be connected with the drug levels of the brain injury areas. The drug reached to the brain injury areas through blood after caudal vein injection immediately, while in intraperitoneal group, the drug was absorbed by the peritoneum firstly, then through the portal vein into the blood cistern into the blood circulation, and finally reached to the brain injury areas with lower concentration than caudal vein injection.

Fas/FasL signaling is an important signaling pathway for cell apoptosis [9]. Fas, an important transduction of cell surface involved in apoptosis, combines with FasL to activate Caspase family to induce cell apoptosis [10]. Caspase family induces apoptosis through mitochondrial pathway and non-mitochondrial pathway, and both pathways eventually induce apoptosis through the activation of Caspase-3 [11], so there are high levels of expression of Fas/FasL and Caspase-3 proteins during cell apoptosis. Our previous study has found that MT could reduce the cerebral ischemia-reperfusion injury by reducing the expression of Fas/FasL and cleaved Caspase-3 protein [12]. In this study, a large amount of Fas+, FasL+ and Caspase-3+ cells were detected in MCAO group, and fewer Fas+, FasL+ and Caspase-3+ cells were observed in the intraperitoneal injection and caudal vein injection group at lesioned side of cerebral cortex. 24 hours and 72 hours after IR, the Fas+, FasL+ and Caspase-3+ cells in intraperitoneal and caudal vein injection group were significantly fewer than those in the MCAO group, which indicated that MT could inhibit the expression of cleaved Caspase-3, Fas and FasL proteins, thus alleviates the ischemia-reperfusion injury via inhibiting the neuronal apoptosis. 7 days after IR, there was no significant difference in the number of Fas+, FasL+ and Caspase-3+ cells between intraperitoneal and MCAO group. And there was fewer Fas+, FasL+ and Caspase-3+ cells in the caudal vein group as compared with the MCAO group. And there were fewer Fas+, FasL+ and Caspase-3+ cells in the caudal vein group than in intraperitoneal group. All results above suggest that the therapeutic effects are...
more obvious in rats after focal cerebral IR in the caudal vein injection as compared with the intraperitoneal injection.

In conclusion, MT therapy by both intraperitoneal and caudal vein injection were effective in alleviating the grey matter injury in cerebral IR rats; furthermore, MT by caudal vein injection was more effective than by intraperitoneal injection in cerebral IR injury rats. The mechanism may be associated with that MT by caudal vein injection is more liable to inhibit the expression of Fas, FasL and cleaved Caspase-3 proteins, and thus alleviate brain grey matter damage.

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Conflict of interests

All contributing authors have no conflict of interest.

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