Title: Melatonin Attenuates Cerebral Ischemia/Reperfusion Injury Through Inducing Autophagy

Running Title: Melatonin and Autophagy on CI/R Injury

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Abstract

Aim: The aim of this study was to investigate how melatonin administration for 3 days or 7 days following cerebral ischemia injury (CI/R) would affect autophagy, and therefore, survival in neurons of the penumbra region. Moreover, it was also aimed to determine how this melatonin treatment would affect the neurological deficit score and rotarod and adhesive removal test durations.

Material and Method: Focal CI (90 min) was achieved in a total of 105 rats utilizing a middle cerebral artery occlusion model. After the start of reperfusion, the groups were treated with melatonin (10 mg/kg/day) for 3-days or 7-days. On all groups, neurological deficit scoring, rotarod and adhesive removal test were executed during reperfusion. Infarct areas were determined by TTC staining at the end of the 3^{rd} and 7^{th} days of reperfusion. Beclin-1, LC3, p62 and caspase-3 protein levels were assessed using Western blot and immunofluorescence methods in the brain tissues. Moreover, penumbra areas were evaluated by transmission electron microscopy (TEM).

Results: Following CI, it was observed that melatonin treatment improved the rotarod and adhesive removal test durations from day 5 and reduced the infarct area after CI. It also induced autophagic proteins Beclin-1, LC3 and p62 and suppressed the apoptotic protein cleaved caspase-3. According to TEM findings, melatonin treatment partially reduced the damage in neurons after CI.

Conclusion: Melatonin treatment following CI reduced the infarct area and induced the autophagic proteins Beclin-1, LC3 and p62 via inhibiting the apoptotic caspase-3 protein. In addition, reflections of the melatonin treatment started to show on neurological tests from the 5^{th} day.

Keywords: Cerebral ischemia, autophagy, melatonin, neurological deficit score, rotarod test, adhesive removal test

Introduction

Cerebral ischemia (CI) is identified as a form of severe metabolic stress resulting from the lack of oxygen and glucose that occurs when the blood flow to a part of the brain is abruptly interrupted due to blockage of an artery [1]. The cells in the infarct core undergo necrosis and die within several minutes after stroke. In the surrounding region, which is identified as ischemic penumbra, cell death progresses slowly within a course of hours or even days following insult, therefore, there is a valuable time frame for rescuing injured cells, particularly autophagic cells [2].

Autophagy regulates the quantity and quality of organelles and proteins by recycling metabolic substrates and energy sources through lysosomal systems, contributing to the maintenance of cellular stability and genomic stability. It is activated during ischemia to produce energy and support cell survival [3]. Following CI, the balance between cell survival and cell death signals determines the fate of neuronal cells [4]. Basal autophagy is a necessary process for the maintenance of cellular homeostasis and adaptation to various stress conditions [5]. However, while inadequate or defective autophagy can induce ischemic damage in the brain, excessive autophagy can also increase cell death and thus cause neuronal death in ischemic cerebral injury [6]. That is, while suitable autophagic activity is neuroprotective, excessive autophagy generally promotes cell death [2]. Because of this sensitive balance, the role of autophagy in CI is still not fully elucidated and understood [6].

Autophagy is a multistep lysosome-mediated degradation process in which unfolded or misfolded cellular proteins, long-lived proteins and damaged organelles are engulfed by a double-membrane bound structure and degraded following fusion with a lysosome [7]. The most important/critical steps in autophagy are the formation of the vesicle called autophagosome and the selective recruitment of target organelles and proteins to this vesicle [8]. Beclin-1, Microtubule-associated protein-1 light chain 3 (LC3) and p62 are essential autophagy-related proteins involved in the autophagic flux [9]. Beclin-1 regulates the transport of other autophagic proteins to the pre-autophagosomal membrane during autophagosome formation. LC3 exists in two different forms; cytosolic (LC3-I) and membrane-bound (LC3-II). Autophagosome formation is closely associated with the conversion of LC3-I to LC3-II. LC3 directly binds adaptor protein p62, which regulates autophagic degradation of misfolded proteins or aggregates in lysosome. In defective or impaired autophagy, p62 protein accumulates, so the level of p62 is inversely proportional to autophagic activity. Therefore, Beclin-1, LC3 and p62 proteins are widely used as biomarkers to determine the level of autophagy [10].

It has been suggested that neuronal cell death occurs via apoptosis after ischemic brain injury [11, 12]. Caspase-3 is the key factor of apoptosis as it activates DNA fragmentation, by activating endonucleases, resulting in cell death [11]. In addition, there are two binding sites for caspase-3 on Beclin-1; if caspase-3 binds to Beclin-1 and causes its destruction, the cell prefers the apoptosis pathway rather than autophagy [13].

Melatonin plays a significant part in the repair of the damage induced by oxidative stress by inhibiting protein oxidation, lipid peroxidation, mitochondrial dysfunction and DNA damage due to its direct free radical scavenging activity and its contributions to the antioxidant defense system [14]. The administration of melatonin during CI increases Bcl-2 protein level and inhibits cytochrome-c release from mitochondria, reducing apoptosome formation and caspase-3 activation [15]. The knowledge on the role of autophagy in melatonin-mediated protection against CI/reperfusion (CI/R) injury is still limited and extensively controversial [16]. However, it is generally accepted that proper autophagic activity is neuroprotective in CI, while excessive or inadequate autophagy is destructive [17].

The aim of this study was to investigate how melatonin administration for 3 days or 7 days following cerebral ischemia injury (CI/R) would affect autophagy, and therefore, survival in neurons of the penumbra region. Moreover, it was also aimed to determine how this melatonin treatment would affect the neurological deficit score and rotarod and adhesive removal test durations.

Materials and Methods

Ethics statement and animal care

All animal experiments were approved by and performed in accordance with the ethical guidelines of the Inonu University, Faculty of Medicine, Experimental Animal Ethics Committee (Protocol No: 9833). One hundred and five male *Wistar albino* rats, weighing 220-280 g, were used. The animals were housed in a temperature-controlled (22±1 °C) environment with a 12/12-h light/dark cycle with ad libitum access to standard laboratory chow pellets and water. Minimum number of animals required for statistical significance was used for the study, and maximum effort was made to minimize any suffering or discomfort.

Experimental design and groups

A total of 105 rats were included in the study, with 15 rats being in each group (6 rats for Western blot and transmission electron microscopy (TEM), 4 rats for immunofluorescence analysis, and 5 rats to calculate infarct area). All rats were weighed and animals with similar body weights were randomly divided into 7 groups as Sham CI/R, CI/R3, CI/R3+EtOH, CI/R3+Mel, CI/R7, CI/R7+EtOH, CI/R7+Mel (n =15).

All animals underwent three days of pre-training of behavioral tests and then Sham CI (All surgical operations except vessel occlusion were performed) or transient focal CI surgical operations were performed. After 90 minutes of CI, reperfusion was provided to CI/R3, CI/R3+EtOH and CI/R3+Mel groups for 3 days, and to CI/R7, CI/R7+EtOH and CI/R7+Mel groups for 7 days. The melatonin (10 mg/kg) [18] was injected intraperitoneally once a day for three days (group CI/R3+Mel) or seven days (group CI/R7+Mel) 1 hour after initiation of reperfusion. The vehicle (same volume, 0.5% ethanol) [19] was injected intraperitoneally with once daily for three days in the CI/R3+EtOH group, and 7 days in the Sham CI/R and CI/R7+EtOH groups. In addition, neurologic deficit scoring and behavioral tests were performed during reperfusion 24 hours after Sham CI or transient focal CI in all groups.

No rats died during the study period, and at the end of the study there were fifteen animals in each group.

Cerebral Ischemia/Reperfusion model induction

Focal CI was induced by occlusion of the right middle cerebral artery (MCA) using the Koizumi intraluminal filament technique [20]. The animals were anesthetized, and their body temperature was kept constant between 36.5-37 °C during the surgical operation. Briefly, after a midline incision, right common carotid artery (CCA) and external carotid artery were isolated and ligated. A microvascular clip was temporarily placed on the internal carotid artery. Then, a

4–0 nylon monofilament coated with silicon resin was inserted into the CCA through a small incision and gently advanced for a distance of 18-20 mm (until slight resistance was felt) from the CCA bifurcation to occlude the origin of the MCA for 90 min. After 90 min ischemia, the monofilament was withdrawn to initiate reperfusion. The Sham CI/R group underwent the same surgical operation without filament insertion.

Behavioral Tests

Neurological score

The neurological deficit scoring test was performed to evaluate the motor coordination and sensorimotor dysfunction of rats during the 3-day or 7-day reperfusion period after CI. Neurological deficit scoring test of the animals were evaluated 24 hours after the initiation of reperfusion, at the same time every day for three or seven days. The test was performed with the modified Bederson scoring method: no deficit=0, forelimb flexion=1, forelimb flexion plus decreased resistance to lateral push=2, unidirectional circling=3, longitudinal spinning or seizure activity=4, no movement=5. The average score of each animal from three trials was used for statistical analysis [21].

Rotarod test

The rotarod test was performed to evaluate the motor coordination, balance, and grip strength of rats during the 3-day or 7-day reperfusion period after CI. Animals (in all groups) were pre-trained 3 days before the CI operation [22]. The rotarod tests were performed to all animals in the groups at the same times every day for three or seven days, 24 hours after the initiation of reperfusion. The time the rats could stay on the rod was measured by increasing the speed from 4 rpm to 40 rpm within 5 min. The 5 trials were performed for each animal and the mean of all trials was compared and day-to-day. The mean of all trials for each animal was compared day-to-day [23].

Adhesive removal test

The adhesive removal test measuring sensorimotor dysfunction (somatosensory neglect) and motor asymmetry of the rats was performed during the reperfusion period after CI. After the adhesive tapes (1x1 cm^2) were placed on the forelimbs, the time until the animal removed the adhesive tapes was measured in seconds. All animals in the groups were trained for 3 days before surgery. The test was applied to all animals in the groups at the same times every day for three or seven days, 24 hours after the initiation of reperfusion. The time the animals could remove the adhesive tapes from each paw was recorded. All trials were averaged for each animal and compared day-to-day [24, 25].

Measurement of infarct area

At third day and seventh day after reperfusion, the rats decapitated, the frozen brain tissues were sliced into 2-mm-thick coronal slices between the bregma levels of +4 mm (anterior) and −6 mm (posterior). After the brain sections were incubated for 30 min in 1% TTC (2,3,5-triphenyltetrazolium chloride) (Sigma-Aldrich, USA) solution at 37 °C, the normal brain tissues were dyed red, and the infarct tissue appeared white. The infarct areas (%) were calculated according to the following formula: (area of the contralateral hemisphere − non-infarct area of the ipsilateral hemisphere) / (non-infarct area of the ipsilateral hemisphere) ×100 [26, 27].

Dissection of ischemic penumbra area

In this study, the ischemic penumbra area in the cortex was used as shown in Figure 1. First, the brain was divided into three slices, starting 3 mm away from the anterior end of the frontal lobe. The anterior and posterior slices were 3 mm thick. The middle slice was 4 mm thick and was cut 2 mm longitudinally from the midline in the ischemic hemisphere. To separate the core region from the penumbra, a transverse cut was made at the 2 o'clock position to obtain the penumbra in the cortex. Western blot, immunofluorescence and TEM analyzes were then performed using the penumbra area in the cortex [28].

Western blot

The ischemic penumbra tissues from the cortex of six rats in each group were individually homogenized, and lysed with added RIPA Lysis Buffer (ab156034, Abcam, UK) of protease and phosphatase inhibitor cocktail (ab201111, Abcam, UK). Equal amounts of total protein (40 μg) were separated by SDS-PAGE and transferred to PVDF membrane. After blocking with 5% nonfat milk for 1 hour at room temperature, PVDF membranes were incubated with primary antibodies in 5% Bovine Serum Albumin (BSA) overnight at +4°C. After washing with TBST buffer, PVDF membranes were incubated with peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, ab205718, Abcam, UK) in 5% nonfat milk for 1 hour at room temperature. The findings were expressed as the fluorescence densitometry ratio of Beclin-1 (2 μg/ml, ab62557, Abcam, UK), LC3-I/II (1:500, ab128025, Abcam, UK), p62 (2 μg/ml, ab91526, Abcam, UK), and cleaved caspase-3 (1:500, ab49822, Abcam, UK)/β-actin (1:1000, ab8226, Abcam, UK).

Immunofluorescence

Brain tissues of four rats in each group were sliced into three 8-μm thick equal serial coronal sections at the bregma level (0.0 mm), using a freezing microtome (Leica CM1850, Germany).

After the brain sections were washed with PBS, the sections were permeabilized with PBST for 1 h at room temperature. The sections were then incubated with primary antibodies Beclin-1 (20 μg/ml, ab62557, Abcam, UK), LC3 (2 μg/ml, ab128025, Abcam, UK), p62 (20 μg/ml, ab91526, Abcam, UK), and cleaved caspase-3 (1:500, ab49822, Abcam, UK) overnight at 4°C. After washing with PBS, the sections were labelled with Alexa Fluor ® 488 secondary antibody (1:500, ab150077, Abcam, UK) for 1 h at room temperature in the dark. The sections were washed three times with PBS and counterstained with DAPI (ab104139, Abcam, UK) for 5 min, then rinsed in PBS and sealed with cover slips. The sections were photographed digitally using an upright optical microscope (fluorescent microscope) (NIS-Elements Documentation 5.02 Nikon Instruments Inc., Melville, NY). The Image J Cell Counter plugin was used to mark and count Beclin-1, LC3, p62, and cleaved caspase-3-positive neurons in the penumbra area in the images. After positive neurons were labeled, Image J was utilized to overlay images to measure colocalization. Selected regions from the peri-infarct area in the three sets of cortices from each rat were counted. All counting was performed by two researchers blinded to the study groups.

Transmission electron microscopy

Neurons, glia cells, vascular structures, myelin sheaths, organelles (especially lysosomes), vacuoles and autophagosome were examined by TEM in the penumbra area taken from the cortex of six rats in each group. Cells with smooth axon and dendrite projections, large and prominent nuclei, euchromatic appearance, oval-round shaped and smooth contours were considered as neurons. Heterochromatic cells with small nuclei, perivascular location, and dense cytoplasm were considered as glia. The peri-infarct (ischemic penumbra) tissue samples of 1 mm^3 volume collected from the cortex were fixed in 2.5% glutaraldehyde and postfixed in 1% Osmium tetroxide. Following dehydration by passing through acetone series, brain tissues were embedded in Araldite CY212. The sections of 80-nm thickness were prepared with an ultramicrotome from araldite blocks. Following contrast-enhanced using uranyl acetate and lead citrate, thin sections were examined under Zeiss Libra 120 TEM (Carl Zeiss, Germany) by a researcher blinded to the study groups, and images were captured.

Statistical analysis

While evaluating the findings, the SPSS 25.0 program was utilized for statistical analysis. Normal distribution of the variables was assessed by Shapiro Wilk test. Since the data of neurological deficit scoring, rotarod test and adhesive removal test of the groups were not normally distributed, the differences between the groups were analyzed with the Kruskal Wallis

H test. Then, Mann Whitney U test with Bonferroni correction was used for intergroup comparisons. The Friedman test was used for intragroup (day-day) comparisons for neurological deficit scoring, rotarod test and adhesive removal test. Data were given as median (smallest-largest). The Kruskal Wallis H test was used to evaluate the Western blot, immunofluorescence, and infarct area results of the groups. When significant differences were detected between groups, multiple pair-wise comparisons were made using the Mann-Whitney U test with Bonferroni correction. Findings are given as mean±SEM. P value <0.05 was considered statistically significant.

Results

Melatonin treatment for 7 days can improve motor coordination and sensorimotor dysfunction

Melatonin treatment may not affect neurological score

Following 90 min of CI, the neurological score of the ischemia, vehicle, and melatonin treatment groups increased significantly compared to the sham operation group. The administration of melatonin (10 mg/kg/day) during both the 3-day and 7-day reperfusion periods following 90 min of CI had no effect on improving the neurological deficit score. However, 3-days melatonin (10 mg/kg/day) treatment significantly reduced the neurologic deficit score to the previous day between the 1^{st} and 3^{rd} days ($p<0.05$). The 7-days of melatonin treatment also improved the neurological deficit score between 2^{nd} and 3^{rd} days, 3^{rd} and 4^{th} days, 4^{th} and 5^{th} days, and 5^{th} and 6^{th} days ($p<0.05$) (Fig. 2).

Motor coordination and balance improved with 7 days of melatonin treatment, while 3 days of melatonin treatment had no effect

The rotarod test durations of the ischemia, vehicle, and melatonin treatment groups reduced significantly compared to the sham-operated group. Melatonin administration (10 mg/kg/day) for 3-days had no additional effect on the rotarod test duration. However, during the 7-day reperfusion period, melatonin treatment increased the rotarod test duration on the 5^{th}, 6^{th}, and 7^{th} days ($p<0.05$). In addition, the rotarod test duration of all groups increased significantly compared to the previous day ($p<0.05$) (Fig. 3).

Sensorimotor dysfunction (somatosensory neglect) and motor asymmetry were recovered with 7 days of melatonin treatment, however 3 days of melatonin treatment had no effect

The adhesive removal test durations of the ischemia, vehicle, and melatonin treatment groups increased significantly compared to the sham-operated group. Three days of melatonin treatment had no significant effect on the adhesive removal test time, whereas 7 days of melatonin treatment reduced the adhesive removal test time on days 5, 6 and 7 ($p<0.05$). In addition, the adhesive removal test duration of all groups increased significantly compared to the previous day ($p<0.05$) (Fig. 4).

Both 3-day and 7-day melatonin treatments decreased the infarct area following CI

Infarct areas were determined with TTC staining. No infarct area was observed in the brain of sham-operated rats. In the groups that were reperfused after 3 days, it was 40.25% in the ischemia group and 42.13% in the vehicle group, whereas the infarct area decreased to 28.39%

in the group that received 3-day melatonin treatment ($p<0.05$). In the groups that were reperfused after 7 days, it was 35.76% in the ischemia group and 37.75% in the vehicle group, while melatonin treated group exhibited markedly smaller infarct areas of 22.41% ($p<0.05$) (Fig. 5). During the reperfusion period, when the duration of melatonin treatment was extended from 3 days to 7 days, a further decrease the infarct area was observed.

Melatonin treatment can induce autophagy

Both 3-day and 7-day melatonin treatments can induce autophagy in the area surrounding the ischemic core

To investigate whether melatonin, a potent neuroprotective agent against CI/R injury, might play a role in rescuing damaged neuronal cells by inducing autophagy, we examined the level of autophagy-related proteins (Beclin-1, LC3-II and p62) by Western blot and immunofluorescence analysis. The concurrent increase in both Beclin-1 and LC3-II proteins and a decrease in p62 indicate activation of autophagy.

Following 90 minutes of CI, Beclin-1 protein levels and positive cells were decreased in the penumbra area of both the ischemia and vehicle groups of the 3 and 7-day reperfusion groups. However, both 3 and 7 days of melatonin treatment after CI increased the Beclin-1 protein level and the number of Beclin-1 positive cells in the penumbra area ($p<0.05$) (Fig. 6).

Quantification of LC3-II/I was also performed following reperfusion to determine the level of autophagosomes in the penumbra area of the cortex. At first, the level of LC3-II/I ratios in the penumbra area were decreased, however with 3 days of melatonin treatment, the level of LC3-II/I ratios were increased ($p<0.05$). Moreover, 7 days of melatonin treatment also increased the LC3-II/I ratio compared to the Sham and ischemia groups ($p<0.05$) (Fig. 7). Immunofluorescence findings were similar to Western blot findings. CI decreased the number of LC3-II positive cells in the penumbra area; however, 3-day melatonin treatment increased the number of LC3-II positive cells ($p<0.05$). Likewise, 7 days of melatonin treatment also increased the number of LC3-II positive cells compared to the Sham and ischemia groups ($p<0.05$) (Fig. 7).

The results showed that 3 days after CI, p62 protein levels and positive cells increased in the area surrounding the ischemic core, yet 3-days melatonin treatment significantly reduced the number of high expression and positive cells of p62. Additionally, the p62 protein levels and positive cells, which were increased after CI, significantly reduced at the end of 7 days of melatonin treatment ($p<0.05$) (Fig. 8).

These results indicate that autophagy was suppressed in the penumbra area following CI/R injury but induced by melatonin, which may have provided a neuroprotective effect. In addition, more autophagic proteins can be induced when the duration of melatonin treatment is increased from 3 days to 7 days.

Both 3-day and 7-day melatonin treatments suppressed apoptotic protein cleaved caspase-3

Caspase-3 is the key factor of apoptosis since it activates DNA fragmentation resulting in cell death [11]. Since the cleavage of caspase-3 plays a critical role in apoptosis, caspase-3 activations were assessed by Western blot and immunofluorescence. Following CI, cleaved caspase-3 protein levels and positive cells were increased in the penumbra area of both the ischemia and vehicle groups of the 3 and 7-day reperfusion groups. However, melatonin treatment decreased the cleaved caspase-3 protein level and the number of positive cells in the penumbra area in both durations ($p<0.05$) (Fig. 9). In addition, cleaved caspase-3 protein level and positive cell counts were lower after 7-days compared to 3-days of melatonin treatment. The results showed that melatonin significantly reduced the key apoptotic protein cleaved caspase-3 protein levels and positive cells in the penumbra area.

Melatonin treatment partially reduced the damage in neurons

In the TEM examination of the sections belonging to the Sham CI/R group; neurons, glia cells and vascular structures were observed in normal ultrastructure. Moreover, cytoplasm and organelles were found in normal density and structure in neurons and glial cells. Lysosomes and autophagosomes were detected in the cytoplasm of neurons in the TEM examination of sections of the CI/R3 and CI/R3+EtOH groups. Additionally, lysosomes and autophagosomes showed heterogeneity in electron density and size in neurons. Intracytoplasmic edema in the neurons and damaged degenerated organelle structures were detected in these areas. In the TEM examination of the CI/R3+Mel group, lysosomes and autophagosomes were observed, however, they were slightly smaller than those in the CI/R3 and CI/R3+EtOH groups. Also, intracytoplasmic edema and organelle damage were minimal in neurons. TEM examination of sections belonging to CI/R7 and CI/R7+EtOH groups revealed diffuse intracytoplasmic edema and organelle damage in neurons and glia cells. Vacuoles of different sizes were widely observed. Degenerated organelle structures were detected in and around these vacuoles. Moreover, necrotic degenerated neurons were observed. In the CI/R7+Mel group, few lysosomes and autophagosomes were observed in the cytoplasm of neurons. The myelin sheaths were generally thinner than the Sham group, yet in a smooth-homogeneous appearance.

Moreover, the myelin sheaths were found similar in structure to those in the CI/R3+Mel group (Fig. 10).

Discussion

Melatonin can cross the blood-brain barrier, have anti-inflammatory, anti-oxidative, anti-apoptotic and analgesic properties and may have protective effects against ischemic damage through autophagy [16, 29].

However, studies examining behavioral tests following melatonin injection in a CI/R model are quite limited. In the literature, mostly the tests were performed only once by providing 24-hour reperfusion. Moreover, in studies examining the infarct area following melatonin injection in the CI/R model, the infarct area was determined by providing 24 hours of reperfusion. In 120-min, 90-min, and 60-min CI/R studies, melatonin injection have been shown to improve the neurologic deficit test score [30-36, 18, 37, 16, 38] and the rotarod test duration [32, 36] performed only once at the 24th hour of reperfusion.

Furthermore, in CI/R studies, studies in which reperfusion was provided longer than 24 hours and neurologic deficit scoring and behavioral tests were examined are very limited. In a study on a 60-min CI model, melatonin (20 mg/kg) was administered before the initiation of reperfusion, and it was reported that melatonin improved the neurologic deficit score in the neurologic evaluation performed on the 5th day of reperfusion [39]. In another study, they reported that melatonin (20 mg/kg) administered at the beginning of reperfusion and after 24 hours decreased the neurologic deficit score and adhesive removal test time on the 1st and 3rd days of reperfusion [40]. It has also been stated that melatonin (4 mg/kg/day) administered intraperitoneally for 29 days following 30-min CI increases the rotarod test time on the 7th and 30th days of reperfusion [23]. Furthermore, it was found that the administration of melatonin (5 mg/kg/day) for 7 days after 90 min CI reduced the neurologic deficit score on the 3rd and 7th days of reperfusion and increased the rotatode test time on the 7th day of reperfusion [41]. In addition, the effect of 10 mg/kg melatonin application on the rotarod test to determine motor coordination and the adhesive removal test to determine somatosensory neglect, started to show from the 5th day. Kilic et al. [23], stated that melatonin improved the motor coordination of animals by reducing neuronal cell death after ischemia and stimulating neurogenesis. Considering our results along with these studies, it can be said that after 90-min of ischemia, 10 mg/kg melatonin administration saves neurons with damaged organelles and proteins through autophagy starting from the 5th day, and motor coordination can be gradually regained via stimulated neurogenesis.

In 90-min CI/R studies performed to date, it has been reported that intraperitoneal melatonin injection reduces the infarct area after 24 hours [34, 35, 18]. In one of the CI/R studies in which

reperfusion was provided longer than 24 hours, the infarct area was determined on the 3rd day of reperfusion [40], and in another study on the 5th day of reperfusion, and it was reported that the infarct area was decreased in the groups given melatonin [39]. In a study similar to our study, 5 mg/kg/day melatonin was administered for 7 days after 90-min CI and it was reported that melatonin reduced the infarct area [41]. In our study, the effect of melatonin administration during 3 and 7 days of reperfusion on the infarct area in the brains of animals were examined. It was observed that 3-day melatonin treatment after 90-min CI reduced the infarct area, and 7-day melatonin treatment reduced the infarct area even more significantly. In addition, it can be said that some reduction in the infarct area can be observed as the reperfusion time is prolonged, however, melatonin application leads to a much greater decrease in the infarct area. In this sense, our study is compatible with other studies in the literature. It has been stated that melatonin provides neuroprotection by preventing the release of cytochrome-c from mitochondria, elevating the level of anti-apoptotic protein Bcl-2, and inhibiting caspases, which are pro-apoptotic proteins [42, 43, 40]. According to our results, it can be said that after 90-min of ischemia, 10 mg/kg melatonin administration prevents neuronal cell loss in the penumbra area.

Numerous studies indicate that autophagy plays a protective role in CI [44-46], while others that autophagy plays a role as a cell death pathway in CI [47-49]. Melatonin have been suggested to have an effect on the autophagy pathway in CI, however, it has not been fully clarified in these studies whether autophagy is beneficial or harmful in the post-CI process. Moreover, it has been suggested that melatonin (10 mg/kg) given 7 days before ischemia in the 120 min CI/R model [16] and melatonin (5 mg/kg) given before CI initiation inhibit autophagy at the end of the 24-h reperfusion period [38]. In the 90-min CI/R model, LC3-II/I protein level and LC3 positive cell number were not changed with 5 mg/kg melatonin given at the 60th minute of ischemia, whereas 10 mg/kg melatonin reduced the Beclin-1 protein level, Beclin-1 positive cell count. and the number of autophagosomes [18]. Lin et al., 90 min after creating 50 min CI determined that administration of 20 mg/kg melatonin on day 1 and day 2 of reperfusion decreased the LC3-II/I ratio 28 days after ischemia, however they did not infer as a cell death pathway or cell survival pathway for autophagy [50].

To date, the impact of melatonin on the role of autophagy has not only been studied in CI/R injury, but also in neonatal cerebral hypoxic-ischemia models. In a HI model, administration of a single dose of 15 mg/kg melatonin 5 minutes after hypoxic-ischemia has been shown to reduce apoptotic and necrotic cell death, increase autophagic activation, and play a protective role for melatonin [51].

According to our results, it can be said that 3-day melatonin treatment stimulates autophagy and 7-day melatonin treatment increases autophagy level even further. In addition, it was determined that the level of cleaved caspase-3 protein increased during the 3-day reperfusion period, however, decreased over time during the ongoing reperfusion period. Our results are consistent with the result of Zhang et al. [17] in terms of the comparison of 3 and 7-day reperfusion times. Zhang et al. showed that the LC3-II protein level peaked in the first 5 hours and remained high for 24 hours [17]. In studies, in which melatonin was given and autophagy was examined in CI, only 24-hour reperfusion was performed [18, 16, 38]. However, it may be difficult to differentiate autophagy and apoptosis in terms of cell death pathway in the first 24 hours, because in one study it was stated that neuronal autophagy was induced after 48 hours compared to other organs [52]. Autophagy together with apoptosis can act as a cell death pathway when physiological stress conditions exceed the adaptive stress conditions of the cell. Therefore, when autophagy is examined in the first 24 hours in CI, it can be seen as a cell death pathway. One of the reasons why it is not possible to explain whether autophagy is a cell death pathway or a cell survival pathway in CI may be that the area where autophagy is examined generally includes the infarct area. However, the main part of autophagy to be examined is the penumbra area since the infarct area includes the core region and these cells already choose the death pathway. However, appropriate autophagic activity in cells in the penumbra can ensure the continuation of cellular life by eliminating damaged organelles and cellular parts instead of leading the cell to death.

Recently, a complex interaction between autophagy and apoptosis has been suggested that they may be sharing molecular inducers and regulatory mechanisms, and that autophagy can modulate caspase-mediated apoptosis [53, 54]. According to our results, levels of Beclin-1 and LC3 were increased, and p62 and cleaved caspase-3 were decreased. In addition, during transmission electron microscope examinations, intracytoplasmic edema in the neurons of the CI groups and damaged degenerated organelle structures in the edema areas were found, while this situation was partially improved in the groups given melatonin.

It has been reported that induction of autophagy is more advantageous than its inhibition in preventing neuronal damage, and that induction of autophagy benefits neuronal survival following CI [17]. Our findings showed that autophagy could be induced protectively after CI in relation to the melatonin treatment duration, and the infarct area could be reduced.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

U.Y.: conceptualization, formal analysis, investigation, methodology, project administration, writing–original draft, and writing–review and editing. K.T.: formal analysis, and methodology. M.G.: formal analysis, and methodology. A.K.: formal analysis, and methodology. S.S.: conceptualization, formal analysis, investigation, methodology, project administration, supervision, writing–original draft, and writing–review and editing.

Data Availability Statement

All data generated and analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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