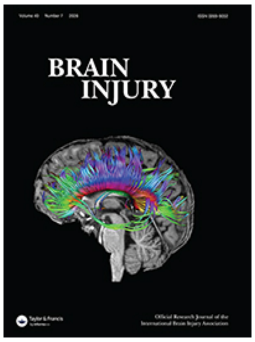

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The protective effects of melatonin postconditioning in cerebral ischemia may be mediated through the modulation of FUNDC1 and Bnip3 levels

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ABSTRACT

Objective: The regulation of mitochondrial bioenergetics – as one of the endogenous defense mechanisms against ischemia-reperfusion (IR) injury – has been considered promising. This study aimed to determine which mitophagy-related signaling pathways (parkin, Bnip3, or FUNDC1) mediate the protective effects of postconditioning (PostC) and melatonin, both of which enhance the intrinsic defense capacity of cerebral tissue. In addition, microRNA-137 and microRNA-145, as well as serum asprosin, a novel glucogenic adipokine, levels were analyzed in cerebral IR injury.

Method: Rats were divided into four groups: control (sham), IR, IR+PostC and IR+Mel(n:10). After 90 minutes of occlusion, PostC was performed at the onset of reperfusion in three cycles of 30-sec reperfusion, followed by 10-sec ischemia.

Results: All parameters involved in mitophagy pathways increased with IR in cerebral cortex, and serum asprosin level decreased. Parkin and PINK1 levels did not change due to the treatments, while the FUNDC1 and Bnip3 levels decreased and serum asprosin levels increased significantly compared to IR. MicroRNA-137 and microRNA-145 decreased, although treatment partially restored the levels of these microRNAs.

Conclusion: Increased expressions of parkin/PINK1, FUNDC1 and Bnip3 may suggest that all mitophagy pathways are activated by cerebral IR. Melatonin PostC may protect the cerebral tissue by inhibiting BNİP3- and FUNDC1-mediated mitophagy.

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Introduction

Stroke is one of the leading causes of death and disability worldwide (1). Ischemic stroke manifests as a disturbance in the blood flow of cerebral tissue and impairs neuronal functioning (2). Brain tissue is extremely sensitive to blood flow changes, and the therapeutic window required to prevent reversible ischemic injury from turning into necrosis is narrow ((3).

Paradoxically, the reperfusion of ischemic tissue may lead to the death of tissue cells that are still alive and salvable with an excessive increase of reactive oxygen species (ROS) and the release of pathological mediators. This chain of events is referred to as ischemia-reperfusion (IR) injury. To maximize the effectivity of IR injury treatments, it is of great importance that they are administered as soon as possible after the onset of symptoms (2). Thus, it is considered necessary to understand the brain's own endogenous protective strategies for neuroprotection (4). Studies have shown that the brain can adapt to adverse events in sublethal doses and to improve cell resistance when exposed to damage (5). The ischemic postconditioning (PostC) principle involves applying short cycles of ischemia during reperfusion to trigger endogenous protective mechanisms following prolonged ischemia.

Aside from classical ischemic PostC studies, pharmacological PostC works are also ongoing. Pharmacological PostC represents the use of pharmacological agents, which create similar effects instead of short ischemic periods (6). Melatonin is a hormone that can directly scavenge ROS produced during normal metabolism in mitochondria and promote the activity of antioxidant enzymes (7). Melatonin treatment reduces neuronal loss, infarction volume in cerebral IR injury and inhibits the loss of mitochondrial membrane potential and the activation of caspase-3 (8).

The regulation of mitochondrial bioenergetics – as one of the endogenous defense mechanisms against IR injury – has been considered promising (9). Nonfunctional and unnecessary accumulation of mitochondria poses a risk to cells and organisms and may lead to neurodegenerative disease (10). Mitophagy controls the quality and quantity of mitochondria by eliminating dysfunctional or excess mitochondria, creating ROS and leading to cell death (9). Insufficient or excess mitophagy may promote cell injury and death (11). Mitophagy occurs through three main signaling pathways: parkin/PTEN-inducible kinase-1 (PINK1), BCL2-interacting protein-3 (Bnip3) and FUN14-domain containing-1 (FUNDC1).

MicroRNA(s) are small non-coding RNA molecules widely distributed within cells that regulate gene expression, with a particularly important role in mitochondrial function (12).

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It has been reported that the enrichment of microRNAs within mitochondria following cerebral ischemia or hypoxia may contribute to ischemia – reperfusion injury (13). Furthermore, MicroRNA(s) have recently been shown to modulate the translational activity of the mitochondrial genome and to regulate the expression and function of proteins (14). MicroRNA

Asprosin is a new glucogenic adipokine and regulates energy homeostasis. It prevents cell death, increases mitochondrial respiration due to hypoxia (15) and protects from oxidative stress-induced apoptosis by activating the ERK1/2-SOD2 pathway in myocardial mesenchymal cells (16).

This study aimed to investigate which pathway(s) of mitophagy (parkin, Bnip3, FUNDC1) provide protective effects in PostC and melatonin treatments. Also, as a damage indicator, expression of brain natriuretic peptide (BNP), neuron-specific enolase (NSE), antioxidative nuclear factor erythroid 2–related factor-2 (Nrf2), serum asprosin levels and some microRNAs (−93, −137, −145 and −181a) which are considered to be associated the above biochemical parameters were investigated.

Materials and methods

Experimental groups

Forty healthy male Sprague-Dawley rats (250–280 g) were maintained in accordance with the Helsinki Declaration, at the Experimental Research Center, Fırat University (Elazig, Turkey) and housed under standard laboratory conditions (Firat University (Elazig, Turkey)/12-h light/dark cycles). Experimental studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The animals consumed the commercial pellet and water, ad libitum. The rats were divided into four groups, each consisting of 10 animals : control, IR, IR +PostC and IR+Mel groups (Figure 1). The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (17) and approved ethically by the Firat University Local Committee on Animal Research (2018/47).

IR procedure

The rats were anesthetized with ketamine hydrochloride+xylazine administered intraperitoneally. Ischemia was induced using the intraluminal filament method of the middle cerebral artery (MCA), as applied by Melani et al. (18) An arteriotomy was performed using microsurgical scissors approximately 3 mm proximal to the bifurcation on the common carotid artery (CCA) after clipping the internal carotid artery (ICA). A poly-L-lysine-coated monofilament with a blunted tip was inserted into the ICA, and the MCA was occluded by carefully advancing the filament about 18 mm until slight resistance was felt. After 90 minutes of occlusion, reperfusion was achieved by removing the suture under anesthesia. PostC was performed at the onset of reperfusion in three cycles of 30-sec reperfusion, followed by 10-sec ischemia. Melatonin solution was administered *i.p* before reperfusion (10 mg/kg). The control group was subjected to anesthesia and sham procedures comparable to those applied to the other experimental groups. The rats were sacrificed by decapitation following 90 minutes of ischemia and 24 hours of reperfusion.

Neurological deficits scores

Neurological deficits were assessed 22–24 hours after MCA occlusion using the following 5-point scoring (19):

1: Contralateral flexion upon lifting the animal by the tail;
2: Normal posture at rest with contralateral flexion;
3: Leaning to the contralateral side at rest;
4: Absence of spontaneous motor activity.

Behavioral tests

Open Field Test: The test was conducted on an apparatus with a base of 80 × 80 cm^2 and divided into 25 equal squares. At the beginning of the experiment, the experimental animal was left in the center of this area and its movements were recorded for 10 minutes. The number of lines the rat crossed was calculated. The assembly was cleaned with a 70% alcohol solution after each use.

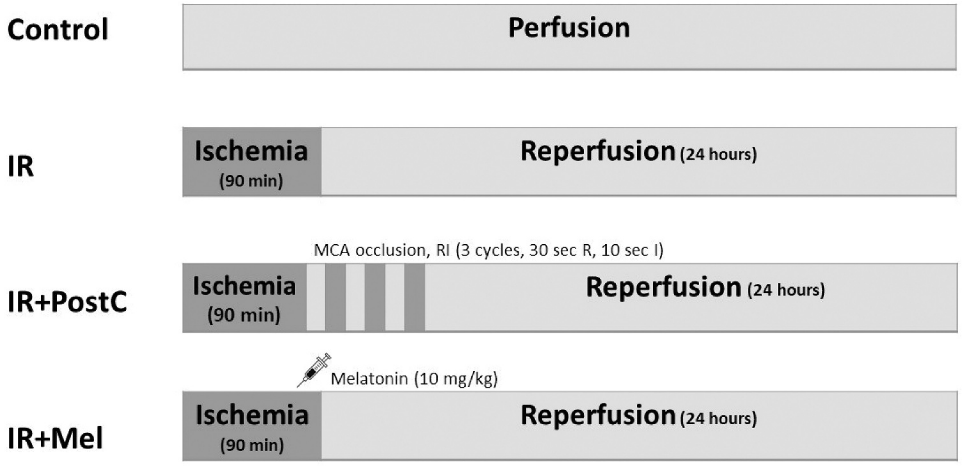

Figure 1. Experimental groups.

Vertical Pole test: The rat was placed on a bar with tape (diameter: 3.0 cm; length: 150 cm); the time it remained on the pole was recorded for a maximum of 180 seconds, those that fell off the bar were assumed to lack motor coordination (20).

Beam walking test: The rat was released at one end of a tape-covered horizontal bar, 50 cm from the horizontal surface, 120 cm long and 5 cm in diameter, and its movement toward the other end was monitored and measured based on the below scoring method (21):

The rat was able;
to walk symmetrically on the beam; 0
to balance and walk on the beam using its unaffected limb preferentially; 1
to balance and walk on the beam relying mostly on the unaffected limb; 2
not to balance on the beam once moved; 3
The rat immediately fell out of the beam; 4.

The test was repeated three times for each rat, and the average scores were calculated for statistical analysis.

4,'6-diamidino-2-phenylindole (DAPI) staining

For evaluating the neuronal injury, the primary somatosensory cortex-barrel area was determined and 18-μm sections were taken in the cryostat apparatus. The sections were pasted on slides, dried and fixed in ethanol at −20 °C for 15 min. After being treated with-phosphate buffer solution (PBS) and 5% bovine serum albumin (BSA), a primary antibody was added, after which PBS washing was performed and the secondary antibody was added, followed by washing with PBS. DAPI was kept in the dark, and after washing/drying in PBS, it was coated with a Gel/Mount preservative liquid and covered with a cover glass. Images were examined and photographs are taken under the ZEISS fluorescence microscope. A density analysis of the relevant regions was made.

Western blot analysis

Relevant antibodies were used for Western blot analysis of tissue parkin, PINK1, FUNDC1, Bnip3, Nrf2 and BNP levels. (ThermoFisher Scientific, catalog number respectively: PA1-751, PA1-16604, PA1-77785, PA5-96388, PA5-68817, PA5-96084). In the Western blot analysis, the penumbral area surrounding the core region induced by MCA ischemia was utilized. The samples were homogenized in the PBS containing the protease inhibitor mixture and the total protein level was then calculated. 50 lg per homogenate was mixed with the sample buffer and, after 5 min of boiling, the samples were separated by SDS-PAGE and electroblotted on nitrocellulose membranes. The membranes were washed in PBS and blocked with 1% BSA for 1 h before primary antibody was applied. Nitrocellulose membranes were incubated overnight at 4 °C with primary antibodies to the proteins and ß-actin (22). Secondary antibodies to proteins were diluted at a concentration of 1:1000 in the same buffer containing 0.05% Tween 20. Densitometric analysis of the bands was analyzed with Image J program.

Quantitative real-time polymerase chain reaction analysis (qRT-PCR)

Cerebral cortex samples were immersed in RNAlater for qRT-PCR. After the RNA was saturated overnight at later, the tissues were stored at −80 °C. Samples were detected in mRNA qRT-PCR. For this purpose, after the tissue samples of each animal were homogenized, the total RNA was isolated from the tissue homogenate using an appropriate RNA isolation kit. The microRNA levels were demonstrated by PCR using the relevant kits and normalized with 'house-keeping' genes. A qRT-PCR analysis of each mRNA was performed for the different experimental groups in the experimental setup. Briefly, 1 μg of total RNA was subjected to qRT-PCR, for which cDNA synthesis was carried out first, after which a qRT-PCR reaction using primers specific to the target mRNAs was performed. (ThermoFisher Scientific, catalog number: microRNA-93:P200117000E09, −137:P200117000E07, −145: P191113004E09,-181a:P200108003c05)

ELISA analysis

Serum asprosin levels were measured with ELISA based on the manufacturer' protocol (Cat. No: 96T-ABBEXA-ABX585287, Abexa).

Statistics

Data were presented as arithmetic mean±SD. When $p < 0.05$, the difference between the groups reached statistical significance. The normality analysis was evaluated using the Shapiro–Wilk test. Parametric tests were applied as raw data provided normality and variance homogeneity tests. These tests were one-way independent measure ANOVAs and Tukey's HSD post-hoc tests.

Results

The mean score of neurological deficits was 2,5 ± 0,97 in the IR group; the treatment methods did not create a significant change on the level when compared to the IR group. (IR+PostC: 2,2 ± 0,67, IR+Mel:2,1 ± 0,82) (Figure 2(a)). When the open field test data were examined; the number of lines crossed by the rats decreased significantly in the IR group (32,30 ± 8,74) compared to the control (135,3 ± 32,74). There was no significant change in IR+PostC (44,60 ± 11,34) and IR+Mel (45,50 ± 12,34) groups when compared to the IR group (Figure 2(b)). In the vertical pole test, when the duration of rats staying on the pole was evaluated, it was decreased significantly in the IR group (23,10 ± 13,98 sec) when compared to the control (148,1 ± 19,97 sec), but there was no significant change statistically in the treatment groups compared to the IR group (IR+PostC: 28,20 ± 12,78 sec, IR+Mel: 33,00 ± 11,09 sec) (Figure 2(c)). The mean of beam walk test scores increased significantly with IR (2,99 ± 0,99), treatment methods did not create a significant change (IR+PostC: 2,7±,95, IR+Mel:2,7 ± 0,82) (Figure 2(d)).

According to the DAPI staining analysis results, when the cell viability of the control was accepted as 100%, the cell

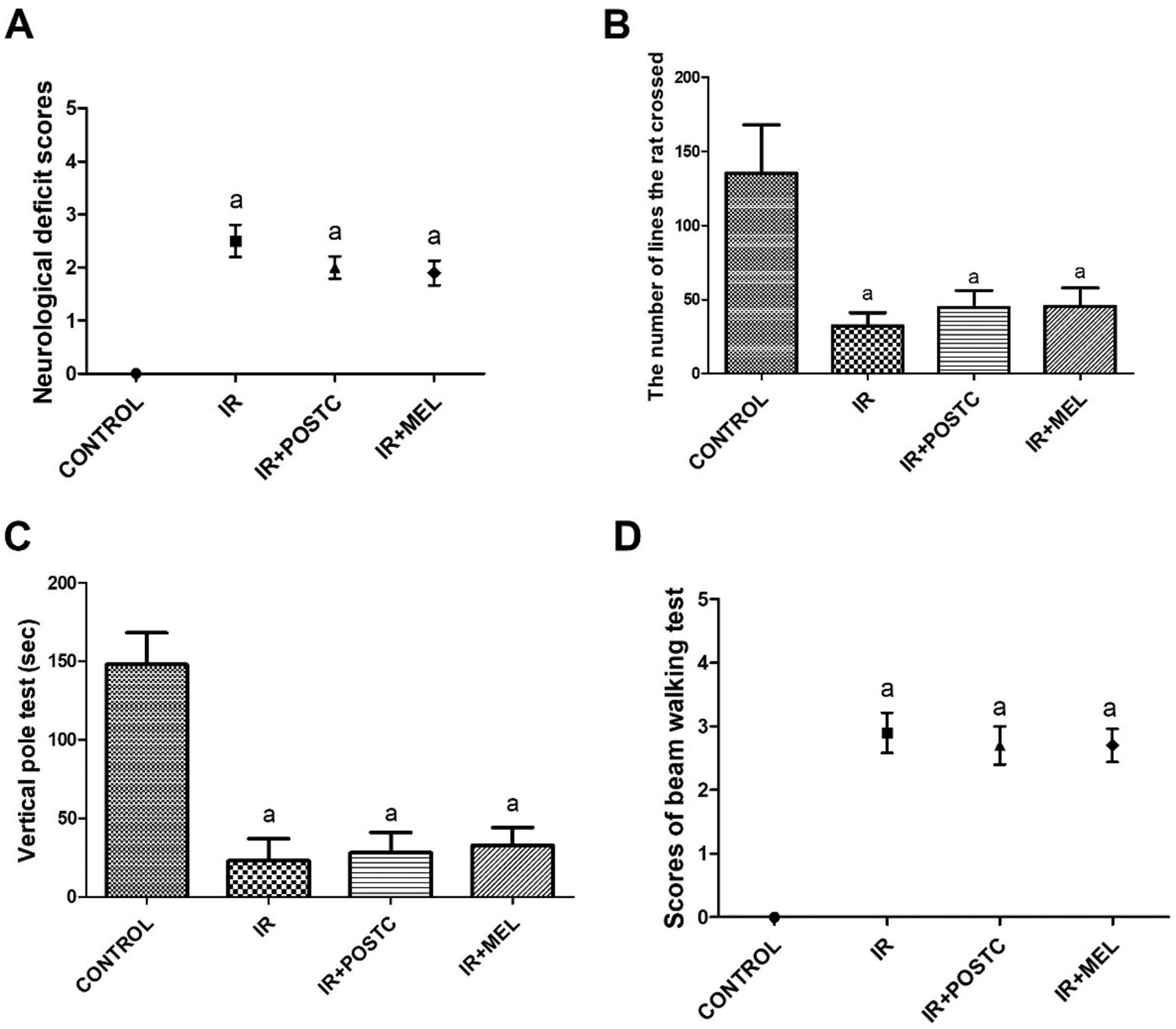

Figure 2. Behavioral tests (n:10, $p < 0.05$). 2a the changes of neurological deficits scores between the groups, 2b the changes of open field test scores between the groups, 2c the changes of the vertical Pole test scores between the groups, 2d the changes of the beam walk test scores between the groups. (a: significant difference compared to control ($p < 0.05$)).

density in the IR group was 54,96 ± 6,04%. The cell density ratio increased significantly more in the PostC (67,90 ± 7,36)- and melatonin (72,8 ± 5,56)-treated groups than in the IR group (Figure 3).

NSE and BNP protein expressions increased significantly in the IR group (133,96 ± 4,95; 238,38 ± 2,13, respectively) compared with the control. The levels of this protein were markedly decreased in IR+PostC (115,14 ± 7,26; 178,29 ± 4,62, respectively) and IR+Mel (105,62 ± 6,86; 179,74 ± 9,24, respectively) groups when compared with the IR group (Figure 4(a, b)). Nrf2 protein expression decreased significantly in the IR group (69,18 ± 2,13) when compared with the control. It increased dramatically in IR+PostC (81,87 ± 2,60) and IR +Mel (81,51 ± 7,21) compared with the IR group. When the changes in the treatment groups were compared with each other, statistical significance was not note (Figure 4(c)).

Parkin and PINK1 protein expressions increased significantly in the IR group (167,84 ± 5,95; 132,83 ± 8,22, respectively) when compared with the control; the changes in the IR+PostC (168,53 ± 2,84; 129,4 ± 4,7, respectively) and IR+Mel (179,04 ± 4,68; 140,05 ± 6,01, respectively) groups were not significant statistically when compared with the IR group (Figure 5(a,b)). Bnip3 and FUNDC1 protein expressions increased significantly in the IR group (135,08 ± 2,52; 169,07 ± 15,92, respectively) when compared with the control, the expressions of these proteins decreased significantly in the IR+PostC (115,89 ± 5,25; 84,96 ± 6,52 respectively) and IR+Mel (85,96 ± 4,63; 88,66 ± 9,26 respectively) groups when compared with the IR group (Figure 5(c,d)).

The microRNA-93 (1,64 ± 0,01) gene expression levels were found to increase, and the microRNA-137 (0,79 ± 0,01), -145 (0,59 ± 0,01), and -181a (0,62 ± 0,01) expression levels were found to decrease with IR injury. MicroRNA-93 levels decreased in IR+PostC (1,53 ± 0,02) and IR+Mel (1,47 ± 0,01) groups compared with the IR group. The increase in the IR+PostC group did not reach statistical significance, the difference in the IR+Mel group was not significant. MicroRNA-137 levels increased significantly in IR+PostC (1,05 ± 0,02) and IR+Mel (1,14 ± 0,01)

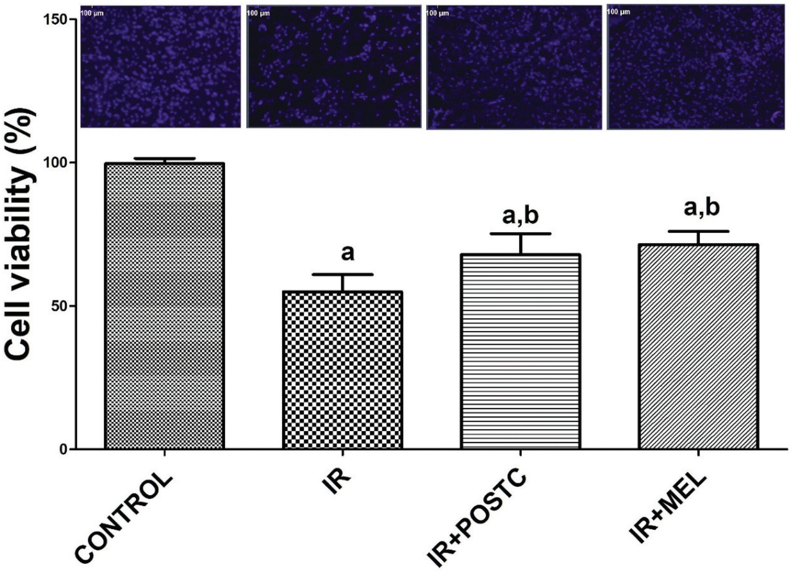

Figure 3. Cell viability. The changes of the cell viability between the groups (a: significant difference compared to control, b: significant difference compared to IR injury (n:10, $p < 0.05$)).

compared with the IR group. The increase in the IR+Mel group was greater than in the PostC group. microRNA-145 levels increased in IR+PostC (0,65 ± 0,01) and IR+Mel (0,79 ± 0,01) groups. Among the treatment groups, the increase in the IR +Mel group was statistically significant. MicroRNA-181a levels tended to increase in IR+PostC (0,64 ± 0,01) and IR+Mel (0,70 ± 0,1) groups when compared with the IR group (Figure 6(a,d)).

The serum asprosin level decreased significantly in the IR group (1,17 ± 1,03) when compared with the control group (6,59 ± 2,11). It increased dramatically in IR+PostC (6,21 ± 1,44) and IR+Mel (4,08 ± 1,72) compared with the IR group (Figure 7).

Discussion

In the present study, the expressions of parkin-PINK1, Bnip3 and FUNDC1, which are involved in different signaling pathways of mitophagy, increased significantly with IR, while the levels of parkin and PINK1 were maintained with PostC and melatonin, and the levels of Bnip3 and FUNDC1 were significantly decreased when compared to IR. The microRNA-93 levels increased with IR, while microRNA-137, -145 and -181a levels decreased. The administered treatments restored the mentioned microRNA levels, albeit partially. The serum asprosin decreased significantly with IR injury and increased in PostC and melatonin. There was an increase in BNP and NSE expressions and a decrease in Nrf2, the treatment groups inhibited these changes.

In the present study, we employed a set of behavioral assays commonly utilized to assess motor function in rodents, including the neurological deficit test, open field test, vertical pole test, and beam walking test. While IR injury was associated with a significant reduction in motor coordination, no significant improvement was observed with melatonin or PostC treatments. This outcome may be explained by the study design, as the animals were sacrificed 24 h after the onset of reperfusion to allow the assessment of alterations in early biochemical parameters. This duration may have been insufficient for the biochemical improvements to translate into measurable clinical manifestations. Similarly, in models of subarachnoid hemorrhage – induced brain injury, isoflurane PostC failed to produce significant changes in neurological scores or motor coordination at 24 h post-surgery; nevertheless, a significant improvement in neurobehavioral function was evident at 48 h (23).

NSE, a neuron-specific biomarker with neurotrophic and neuroprotective activities, is employed as a sensitive marker of neuronal injury in both traumatic brain injury and acute ischemic stroke (24,25). In experimental models, it has been reported that the NSE level increases in MCA occlusion and is positively correlated with infarct tissue volume (26). The present study found that NSE levels that increased with IR injury decreased significantly with melatonin and PostC administrations. Consistent with these observations, fluorescence microscopy analysis after DAPI staining showed reduced cellular labeling in the IR group; however, treatment protocol groups displayed enhanced dye incorporation, suggesting a potential protective or restorative effect. BNP, another indicator of neuronal injury, exhibited a pattern parallel to that of NSE and DAPI staining, with levels rising after occlusion but significantly declining in the treatment groups, thereby supporting the neuroprotective potential of the applied interventions. Similarly, it has been shown that the ischemic zone in the brain may be a potential source of plasma BNP (27,28). NSE and BNP levels may increase in IR to activate survival pathways and provide integrity against acute stress.

Nrf2 stimulates the expression of various antioxidants and detoxifying enzymes (30). It has been reported that the pharmacological activation of Nrf2 can protect cells lacking PINK1 levels from dopamine toxicity (31). It has also been shown that the activation of the Nrf2/heme oxygenase-1 (HO-1)-mediated HIF1α/Bnip3 pathway can alleviate hypoxia-induced apoptosis (32). In mice, microRNA-93 antagonist microRNA treatment has been shown to reduce infarct volume, neural apoptosis and neurological deficits via the Nrf2/HO1 pathway (33).

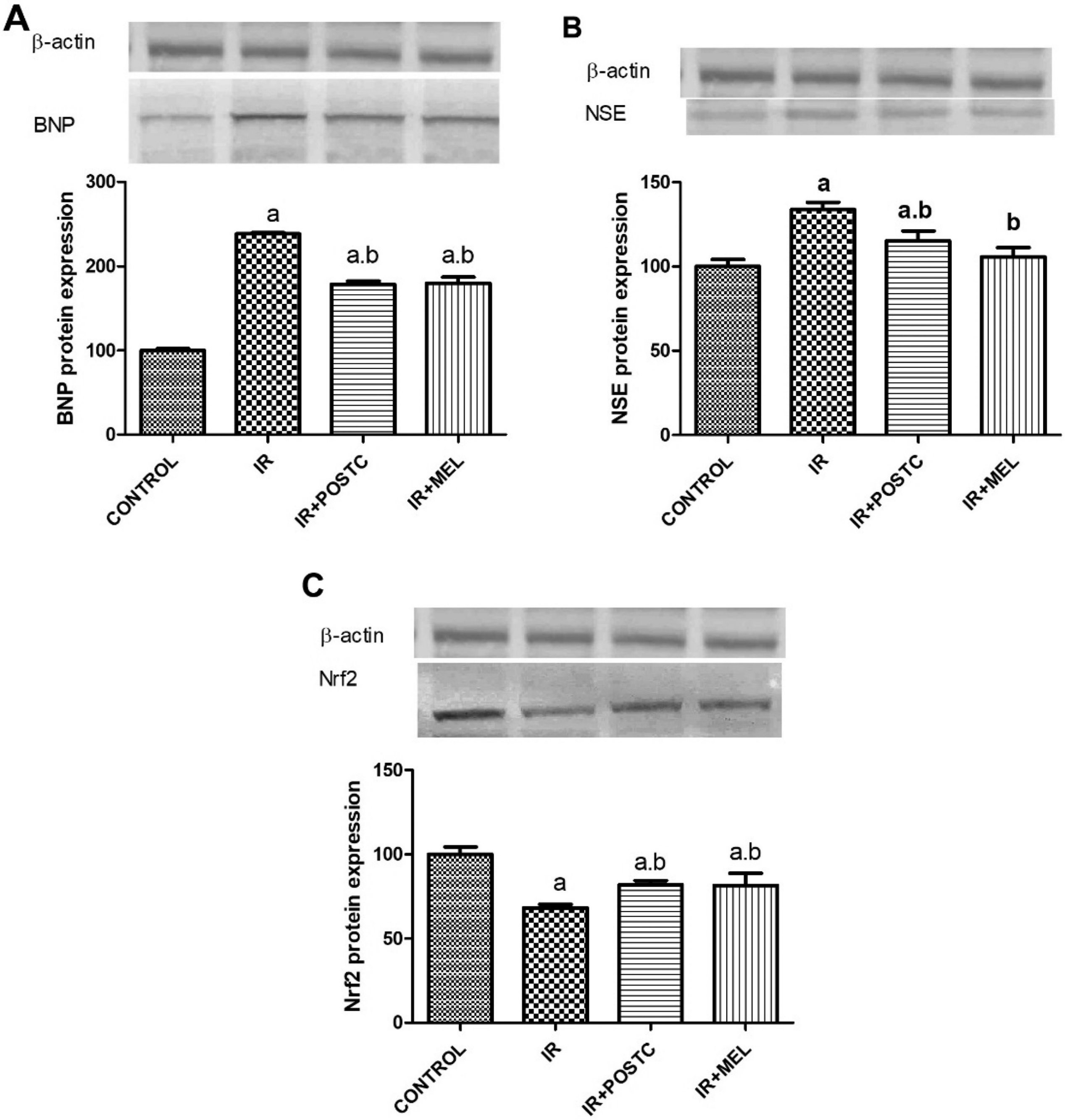

Figure 4. A the changes of the BNP protein expressions, 4b the changes of the NSE protein expressions, 4c the changes of the Nrf2 protein expressions. (a: significant difference compared to control, b: significant difference compared to IR injury (n:10, $p < 0.05$)). (A representative image of the actin loading control, which is identical for all blots as the same samples were used, is shown for all panels.).

MicroRNA-93 may regulate mitochondrial clearance in the presence of stress by directly affecting Nrf2, which, in turn, may directly affect different mitophagy pathways. In our study, Nrf2 levels were reduced following IR injury but increased with melatonin and post-conditioning treatments. Such elevation may facilitate the initiation of PINK1-mediated mitophagy while simultaneously attenuating BNİP3-mediated apoptosis.

Parkin and PINK1 constitute a specific pathway of mitophagy. Parkin, a ubiquitin ligase family member, translocates into depolarized mitochondria in mitochondrial injury. PINK1 is parkin’s upregulator and accumulates in the outer membrane of mitochondria in mitochondrial depolarization (29,34). It has been reported that activating transcription factor-4 – one of the endoplasmic reticulum stress response effectors – suppresses nod-like receptor protein-3 inflammasome activation through a parkin-dependent mitophagy (35), and that the activation of parkin inhibits apoptosis by ubiquitinating voltage-dependent anion-selective channel-1 (36). It has also been shown that the deletion of uncoupling protein-2 one of the internal mitochondrial membrane proteins – induces mitophagy by increasing PINK1 levels (37). Parkin and PINK1 have been reported to reach their maximum level 24 h after MCA occlusion in rats (30). Parkin and PINK1 have

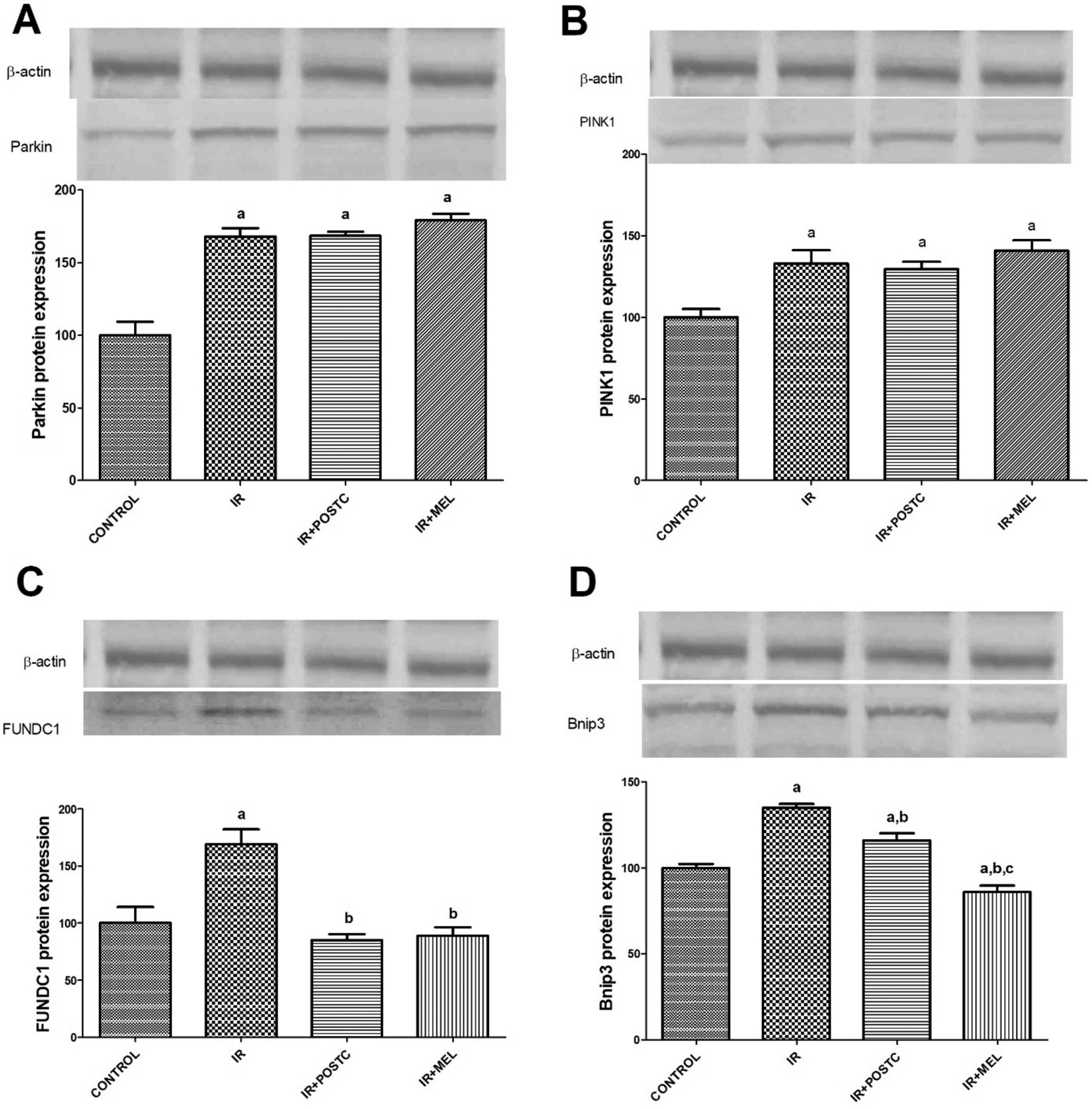

Figure 5. A the changes of the parkin protein expressions, 5b the changes of the PINK1 protein expressions, 5c the changes of the FUNDC1 protein expressions, 5d the changes of the Bnip3 protein expressions (a: significant difference compared to control, b: significant difference compared to IR injury, c: significant difference compared to PostC (n:10, $p < 0.05$)) (A representative image of the actin loading control, which is identical for all blots as the same samples were used, is shown for all panels.).

been reported to reach their maximum level 24 h after MCA occlusion in rats, but that triiodothyronine PostC further increased PINK1 and Parkin protein expressions (31). In the present study, Parkin and PINK1 expressions increased significantly with IR, and treatment administrations ensured the maintenance of these levels.

Bnip3 usually is low in the central nervous system but can be induced in hypoxic conditions (38). It has been reported that silencing the Bnip3 gene is neuroprotective in neonatal brain hypoxia (39), Similarly, while Bnip3 excretions increased with IR injury in our study, it decreased significantly with PosCc and melatonin. Bnip3 integrates apoptosis and mitophagy signaling in different signaling domains, and so crosstalk between mitophagy and apoptosis may affect neuronal cell death during a stroke (40). Increased Bnip3 levels increase during mitophagy under physiological conditions, while it may occur due to apoptosis in pathological conditions such as hypoxia (41).

FUNDC1 is a mitophagy receptor that regulates mitochondrial turnover by interacting directly with light chain-3 (LC3) (42). The activation of FUNDC1 supports mitophagy, thereby protecting nerve cells from apoptosis due to hypoxia (43). Similarly, the depletion of FUNDC1 induces apoptosis and to increase cell sensitivity to ionizing radiation (44). In our study,

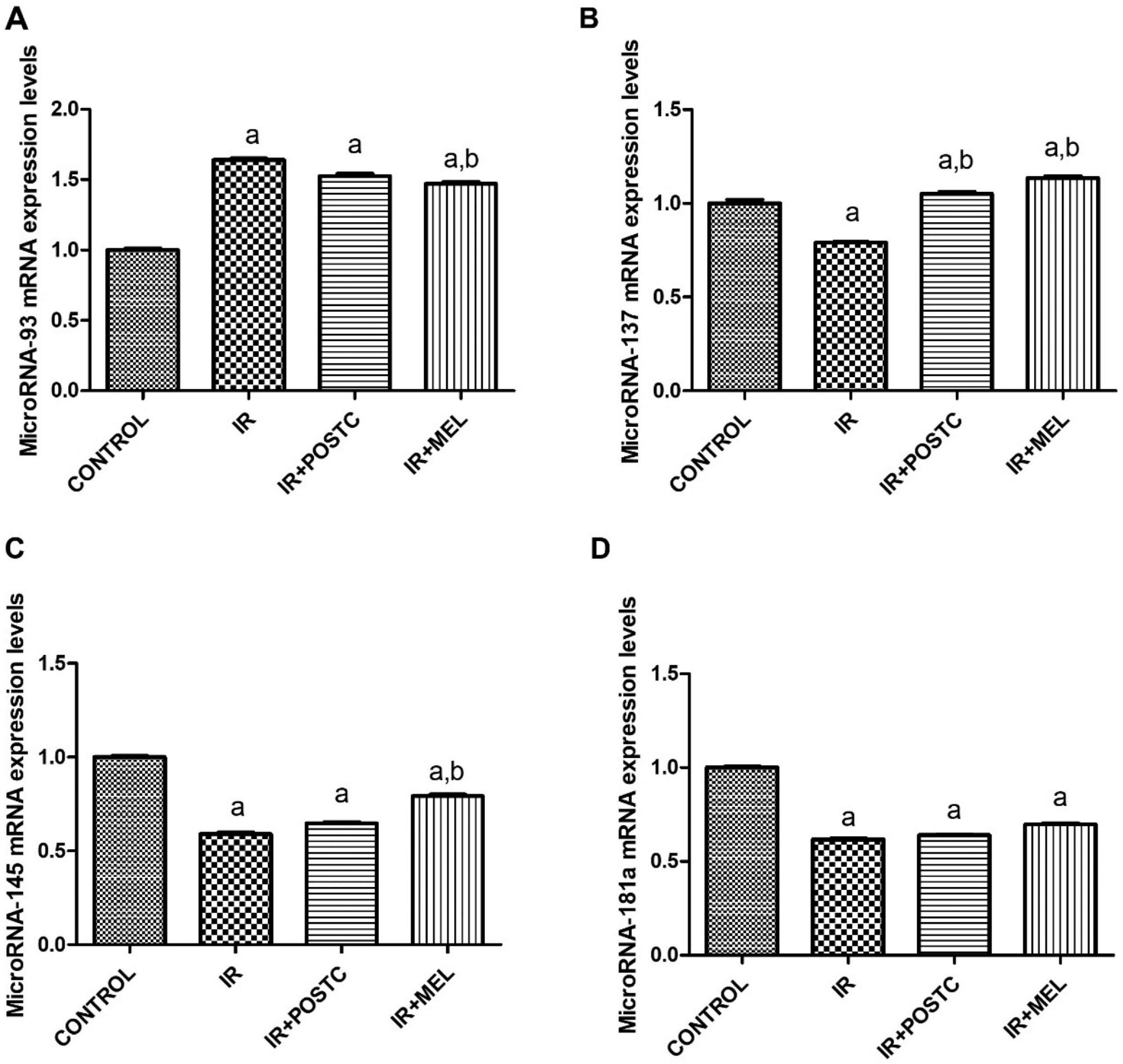

Figure 6. The changes of the microRNA-93 mRNA expressions, 6b the changes of the microRNA-137 mRNA expressions, 6c the changes of the microRNA-145 mRNA expressions, 6d the changes of the microRNA-181a mRNA expressions (a: significant difference compared to control, b: significant difference compared to IR injury (n:10, $p < 0.05$)).

FUNDC1 expression, which increased with IR injury, decreased significantly with the treatments. Similarly, the renoprotection of preconditioning was abolished in FUNDC1 knockout mice (42).

In the present study, all mitophagy pathways were activated in an IR injury, and the parkin-PINK1, Bnip3 and FUNDC1 levels increased. In contrast, the parkin/PINK1 levels did not change with PostC and melatonin treatments, while the Bnip3 and FUNDC1 levels decreased and the nuclear structure was preserved. Although the present study identified no increase in parkin and PINK1 levels attributable to treatment, when evaluated together with the data of other studies, it can be suggested that parkin/PINK1-dependent mitophagy supports survival, that an increase in FUNDC1-induced mitophagy protects cells from apoptosis, and that the increase in Bnip3 levels resulting from apoptosis may cause cellular damage.

MicroRNAs, which regulate target gene expression at a posttranscriptional level, play a critical role in the pathophysiology of ischemic injury by mediating such processes as oxidative stress and autophagy (45). In this study, while microRNA-93 levels increase significantly with IR; microRNA-137, −145 and −181a levels were significantly decreased. No significant change was observed in microRNA-181a levels with treatments. MicroRNA-137 levels increased significantly with both the treatments, while the change in microRNA-93 and −145 levels was significant only in the melatonin group. In the PostC group, significance may not be reached due to the mechanical procedure involved applying the cycles. Moreover,

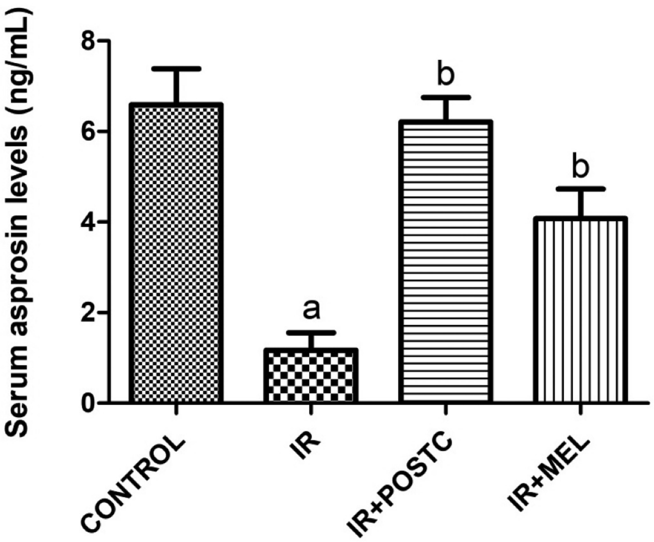

Figure 7. The changes of the serum asprosin levels (a: significant difference compared to control, b: significant difference compared to IR injury (n:8, $p < 0.05$)).

due to its high lipophilicity and potent antioxidant properties, melatonin may have exerted a more potent acute effect on the microRNAs. The downregulation of microRNA93 has been shown to increase Nrf2 expression and to inhibit the inflammatory toll-like receptor-4/NF-κB signaling pathway (33). In addition, microRNA-93 inhibits inflammatory responses and cell apoptosis after cerebral IR by downregulating the interleukin-1 receptor-associated kinase-4 expression (46). MicroRNA-93 may play a role in IR physiopathology by regulating antioxidative pathways and inflammation and Nrf2-mediated mitophagy. MicroRNA-137 has been shown to inhibit mitophagy by regulating FUNDC1 posttranscriptionally (47). Furthermore, microRNA-137 accelerates mitochondrial biogenesis by upregulating Nrf2 (48) and prevents oxidative stress by blocking the Src-mediated MAPK signaling pathway (49). microRNA-145 has been shown to inhibit the transcription and expression of Bnip3, leading to the inhibition of mitophagy, while the inhibition of microRNA145 increases mitophagy activity and promotes survival by reducing tumor necrosis factor-α (TNFα)-mediated inflammation damage (50). microRNA-181a has an antioxidant effect by inducing Nrf2 (51). It has been reported that microRNA-181a directly targets parkin, and that its inhibition accelerates the autophagic degradation of the damaged mitochondria (52). When the our results are evaluated together with the data from the literature; the lack of any significant change in Parkin levels during treatments in the present study may result from the microRNA181a levels, which directly target this protein, not responding sufficiently to treatment. MicroRNA-93 may play a role in cerebral IR physiopathology by regulating antioxidative pathways and inflammation and Nrf2-mediated mitophagy. The effects of melatonin and PostC administrations on FUNDC1 may have been mediated through the upregulation of microRNA-137 levels. The reduction of microRNA-145 levels in response to cerebral IR may have mediated the increase in Bnip3 expression, whereas melatonin may have promoted the elevation of microRNA-145 levels and consequently the inhibition of BNİP3.Asprosin is a fairly new molecule and studies are very limited on the brain function. In a study, no significant change has been observed in the brain tissue of diabetic rats, while serum asprosine levels have been significantly decreased (53). Asprosin has promoted apoptosis by inhibiting β-cell autophagy via AMPK-mTOR pathway (54). In another study, asprosin has improved the survival of mesenchymal stromal cells in myocardial infarction by inhibiting apoptosis via the activated ERK1/2-SOD2 pathway (16). Considering that asprosin regulates energy metabolism and mitochondria is the powerhouse of the cell, it may be thought that there may be a close relationship between mitophagy due to mitochondrial dysfunction and asprosin. A recent study has demonstrated that PINK1- and parkin-mediated mitophagy, induced by a high-fat diet, was markedly reduced under asprosin deficiency, whereas asprosin administration reversed this impairment (2). Accordingly, it may be suggested that asprosin is directly associated with mitophagy mechanisms. In our study, asprosin levels decreased with cerebral IR injury but increased with the treatments. The decrease in the level with pathological stress and increase with the treatments may indicate that asprosine can be a protective molecule and may decrease in the lack of energy.

In conclusion, increased expressions of parkin-PINK1, FUNDC1, and Bnip3 may indicate that all mitophagy-signaling pathways have been activated by the cerebral IR. The maintenance of increased IR-induced levels of parkin-PINK1 through treatment may suggest that the activation of this mitophagy pathway supports cellular survival. FUNDC1 mitophagy, which increases with IR injury, may play a role in maintaining cellular integrity by protecting the cell from apoptosis.

Postconditioning and melatonin may have a protective effect by inhibiting Bnip3 levels. The fact that Bnip3-mediated mitophagy is increased by IR but decreased by treatment, may suggest that this pathway mediates reperfusion injury and that its increase through treatment may be limited.

MicroRNA-93 may play a role in IR physiopathology by regulating the antioxidative pathways and the inflammation and Nrf2-mediated mitophagy. Among the microRNAs, microRNA-137 may play an important role in cerebral IR physiopathology by regulating FUNDC1-mediated mitophagy, inflammatory response, and survival pathways; microRNA-145 by affecting Bnip3 expression levels and inflammation; and microRNA-181a by directly targeting Parkin and inducing Nrf2.

BNP levels may increase when aiming to activate survival pathways and providing mitochondrial integrity against acute stress in IR. The significantly decreased NSE levels associated with treatments may contribute to protection by activating the survival pathways, and thereby by regulating mitophagy.

Serum asprosin levels may decrease with MCA occlusion. The decrease in the level may indicate that asprosine can be a protective molecule and decrease in the lack of energy.

Melatonin may be considered a protective and pharmacological postconditioning agent that uses the same mitophagy pathways as postconditioning.

The limitations of the study are as follows: Our study was terminated 24 h after reperfusion; therefore, treatment-related improvements could only be observed at the biochemical level and were not reflected clinically. This constitutes one of the limitations of our study. Another limitation is that the efficacy of treatments on mitophagy pathways can be verified through the administration of a mitophagy inhibitor agent. This limited aspect has been planned to be addressed in our future study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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