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Asprosin improved neuronal survival by suppressing apoptosis and enhancing the activity of the autophagy pathway in the MCAO model in rats

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Abstract. – OBJECTIVE: Cerebral ischemia (CI) is a condition in which metabolic stress increases when blood flow is interrupted in a part of the brain, resulting in oxygen and glucose deprivation. It is known that asprosin (Asp), secreted from adipose tissue during fasting, has an effect on some metabolic processes such as apoptosis, autophagy, and glucose metabolism. This study aimed to explain which of the cell death/survival Asp induces in the CI/reperfusion model.

MATERIALS AND METHODS: In the study, 48 male Wistar Albino rats were divided into 6 groups: Sham, CI, Asp+CI, CI+Asp, CI+Asp+3-MA, and Asp+CI+3-MA (n=48). CI was created using the intraluminal filament technique for 60 minutes, autophagy inhibitor 3-MA (15 mg/kg/day) and Asp (1 µg/kg/day) injections were administered 3 days before or 3 days during reperfusion. Beclin-1, ATG5, ATG7, p62, Bcl-2, Bax, active-caspase-3, and active-caspase-9 protein levels from brain tissues were determined by the Western-Blot method. The infarct area was determined by triphenyl tetrazolium chloride (TTC) staining. The Kruskal-Wallis' test was used to compare differences between groups. $p<0.05$ was considered statistically significant.

RESULTS: Compared to the Sham group, the increase in ischemic area and the decrease in Beclin-1, ATG-5, ATG-7, Bcl-2, Bax, active-caspase-3 and active-caspase-9 levels in the CI groups are statistically significant ($p<0.05$). The increase of Beclin-1, ATG-7, Bcl-2, and Bax levels in the Asp groups is statistically significant compared to the CI group ($p<0.05$). When Asp+CI groups and CI+Asp groups are compared, an increase in Beclin-1 levels in the Asp+CI group and the increase in Bcl-2, Bax, active-caspase-3/9 and ATG-5 levels in the CI+Asp groups are statistically significant ($p<0.05$).

CONCLUSIONS: Asp has protective and therapeutic effects against CI/R damage. While applying Asp before ischemia activates the autophagy pathway more, applying it after ischemia protects the neuronal death/survival balance by activating the apoptosis pathway more.

Key Words:
MCAO, Cerebral ischemia, Asprosin, 3-MA, Autophagy, Apoptosis.

Introduction

Stroke is the temporary or permanent impairment of a region or the entirety of the brain, resulting from blockages or hemorrhage in the vessels supplying the brain[1]. Stroke is a leading cause of morbidity and mortality worldwide. It ranks as the second leading cause of death and is a primary contributor to acquired adult disability[2]. Approximately 80% of all stroke cases are ischemic in nature, and cerebral ischemia (CI) is defined[3] as the sudden interruption of blood flow in a vessel due to occlusion, resulting in oxygen and glucose deprivation, causing excessive metabolic stress in a part of the brain. Vascular occlusion leads to oxygen and energy deprivation, followed by the formation of reactive oxygen species, glutamate release, intracellular calcium accumulation, and induction of inflammatory processes[4]. Reperfusion, on the other hand, refers to the revascularization and oxygenation of tissues or organs exposed to ischemia. Paradoxically, reperfusion of ischemic tissue or organs can result in more severe damage compared to the damage caused by ischemia alone[5]. This series of events, known as the ischemic cascade, leads to irreversible tissue damage (infarction). Two main approaches have been developed to treat ischemic stroke: neuroprotection and reperfusion.

Various experimental animal stroke models[6] have been developed to investigate stroke pathophysiology, therapeutic approaches, and mimic processes. Although human ischemic stroke formation, causes, and anatomical localization

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are highly diverse, experimental ischemic stroke models offer more precise analyses as they are highly repeatable, well-controlled, and standardized. Molecular, genetic, biochemical, and physiological research often requires invasive direct access to brain tissue, making animal models essential for evaluating stroke pathophysiology and drug effects. The formation of endothelial plaques is one of the primary causes of ischemic damage in mice with a genetic predisposition to type 2 diabetes mellitus[7]. The choice of an appropriate animal stroke model may significantly influence the success of preclinical stroke research for new therapeutic developments. The similarity of rat cerebral vascular structure to that of humans, ideal body size for easy monitoring of physiological parameters, and suitability for brain fixation procedures make rats preferable for stroke research. The most common method for focal ischemic stroke is the intraluminal filament occlusion of the middle cerebral artery (MCAO), which has been used in over 40% of stroke studies[8].

The most critical factor for neuronal survival after a stroke may be the activated defense mechanisms rather than the ischemic process itself. Developing protective systems for individuals at high risk of stroke may be more crucial in approaches aimed at reducing treatment costs. Activating pre-defense mechanisms against neuronal damage may also be effective in protective treatments against other neuropathological diseases[9].

As an alternative to reduce cerebral damage, various antioxidant treatments with variable results have been suggested[10]. Asprosin (Asp) is secreted from white adipose tissue, known for secreting adipokines such as leptin and adiponectin[11]. The majority of studies on Asp in the literature are aimed at determining Asp levels in different disease groups, and most of them are focused on revealing the gluconeogenic and orexigenic properties of Asp[12]. In a study by Wang et al[13], plasma Asp levels were reported to be significantly higher in individuals with impaired glucose regulation and newly diagnosed type 2 diabetes compared to those in the normal glucose regulation group. A study[14] on mouse insulinoma cells revealed that Asp has effects on autophagic parameters. Recent studies in literature have shown that Asp may be effective in the treatment of various pathological processes associated with neuron damage and in the activity of protective pathways. Asp's neuroprotective efficacy has been demonstrated in various experimental neurological studies[15], showing its effectiveness on apoptosis and autophagy pathways, in addition to its antioxidant activity. However, its effectiveness on these pathways in neurological damage due to ischemia/reperfusion (I/R) has not been studied yet. This study aims to shed light on the relationship between Asp's effectiveness and these pathways, providing a better understanding of its activities in brain tissue in this neuropathophysiology.

Materials and Methods

Animals and Drugs

A total of 48 adult male Wistar Albino rats (8 weeks old, 300-350 g) were purchased from the Experimental Animal Research Center of Inonu University, Malatya, Turkey. All the animals were handled in accordance with the Guidelines for the Care and Use of Experimental Animals[16]. All animals were randomly assigned to cohorts and approved by the Animal Research Ethics Committee of Inonu University Faculty of Medicine (License No.: 2021/19-3). The rats were housed at 21±2°C with a 12/12-hour light-dark cycle with free access to water and food *ad libitum* with a standard laboratory chow diet. The number of groups and rats in each group (sample size) was determined according to the power analysis based on the values specified. Accordingly, the amount of Type I error (α) was 0.05, the power of the test (1-β) was 0.8, and the effect size was 0.82 (large). While the number of groups was 6, the minimum sample size required to find a significant difference between the groups was at least 5 in each group for Western Blot (WB) analyses and at least 3 different rat tissues from each group for TTC analyses. With 8 animals in each group, the total number was determined to be 48 rats[17].

Group 1 – Sham Group: Animals in this group underwent sham surgery (all surgical operations outside the vascular capacity were performed). Animals in this group received intraperitoneal (ip) administration of 1 ml of visible saline (PS, solvent for Asp, and 3-MA) once a day for 3 days, starting one hour after Sham CI surgery.

Group 2 – CI Group: Animals in this group underwent a 60-minute temporary focal CI followed by reperfusion. Starting 24 hours after the initiation of reperfusion, animals received

ip injections of 1 ml physiological saline (PS, solvent for Asp and 3-MA) once a day for 3 days.

Group 3 – CI+Asp Group: Animals in this group underwent a 60-minute temporary focal CI followed by reperfusion. One hour after the initiation of reperfusion and for the following 3 days, animals received ip injections of 3 µg/kg/1 ml/day dose of Asp[18].

Group 4 – CI+Asp+3-MA Group: Animals in this group underwent a 60-minute temporary focal CI followed by reperfusion. One hour after the initiation of reperfusion and for the following days, 3 days ip injections of 3 µg/kg/1 ml/day dose of Asp and 15 mg/kg/1 ml/day dose of the autophagy inhibitor 3-MA[19].

Group 5 – Asp+CI Group: Animals in this group received ip injections of 3 µg/kg/1 ml/day dose of Asp for three days. On the 6th day, a 60-minute temporary focal CI was induced, followed by reperfusion. Starting one hour after reperfusion initiation and for the following days, 3 days of ip injections of 1 ml physiological saline were given.

Group 6 – Asp+CI+3-MA Group: Animals in this group received ip injections of 3 µg/kg/1 ml/day dose of Asp for three days. On the 6th day, a 60-minute temporary focal CI will be induced, followed by reperfusion. Starting one hour after reperfusion initiation and for the following 3 days, ip injections of 15 mg/kg/day of 3-MA were administered.

Creation of MCAO/R Model

Anesthesia was induced in rats with intraperitoneal administration of 70 mg/kg ketamine and 8 mg/kg xylazine. Throughout the experiment, the body temperatures of the subjects were maintained at a constant range of 36.5-37°C, monitored by a rectal temperature probe. A Laser-Doppler blood flow meter (Moor Instruments, Axminster, Devon, UK) was used for continuous monitoring of regional cerebral blood flow. The focal ischemia model was created by occluding the right middle cerebral artery using the intraluminal filament technique. After 60 min of ischemia, the monofilament was withdrawn to initiate reperfusion. The Sham CI/R group underwent the same surgical operation without filament insertion. At the end of the required period, the rats were sacrificed under anesthesia, and brain tissues were collected. Brain tissues were TTC frozen on dry ice and stored at -80°C under suitable conditions until the day of the analysis for WB analyses.

Measurement of Infarct Area

On the third day following reperfusion, rats were euthanized, and their frozen brain tissues were 6, sectioned into 2 mm thick coronal slices. The brain sections were then incubated in a 1% solution of TTC (2,3,5-triphenyl tetrazolium chloride) (Sigma-Aldrich, USA) at 37°C for 30 minutes. This resulted in normal brain tissues being stained red, while infarcted tissue appeared white. The percentage of infarct areas was calculated using the following formula: (area of the contralateral hemisphere - non-infarct area of the ipsilateral hemisphere)/ (non-infarct area of the ipsilateral hemisphere) × 100.

Western Blot Analyses

We examined the expressions of Bax, Bcl-2, Cas-3, Cas-9, ATG-5, ATG-7, Beclin, and p-62 in the brains of the rats by WB. In WB analyses, the experiments were conducted in accordance with the standardized method established in previous studies[20]. Tissue samples were homogenized using steal beads (Next Advance BBY24M, Inc. Innovative Lab Products for the Life Sciences, USA) in ice-cold lysis buffer (ab156034, RIPA, Abcam, Cambridge, UK) at pH 7.4. Samples were homogenized in RIPA (ab156034, Abcam, Cambridge, UK) buffer supplemented with protease inhibitors (ab201111, Abcam, Cambridge, UK). BCA assays (BCA Protein Quantification Kit ab102536, Abcam, Cambridge, UK) were used to quantify protein concentration. The same amount of protein was loaded into each well, which, depending on the antibody, ranged from 25 to 50 µg. Proteins were resolved by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (1620177, Biorad Life Sciences Research, USA) as described. Following the transfer with a trans-blot turbo transfer system (Thermo Scientific, Waltham, MA, USA), the membrane was blotted for 1 hour in a 5% milk powder solution prepared with TBS (Tris-Buffer-Saline) containing Tween 20 and washed 3 times with TBS/T 10 minutes apart. Then, membranes were incubated with the following primary antibodies: CAS-3 (E-AB-22115, Elabscience, Texas, USA), CAS-9 (E-AB-15091, Elabscience, Texas, USA), Bax (E-AB-66518, Elabscience, Texas, USA), p-62 (E-AB-70387, Elabscience, Texas, USA), ATG-5 (E-AB-10814, Elabscience, Texas, USA), ATG-7 (E-AB- 22151, Elabscience, Texas, USA), Bcl-2 (E-AB- 60788, Elabscience, Texas, USA), Beclin (E-AB- 81447,

Elabscience, Texas, USA), and β-Actin (ab8226, Abcam, Cambridge, UK) in 5% BSA powder overnight at +4°C. Following primary antibody incubation, membranes were washed 3 times with TBS/Tween 20 (935 B, Sigma-Aldrich, USA) for 10 minutes. Then, incubated with peroxidase-conjugated secondary antibody in a 5% milk powder solution prepared with TBS/Tween 20 for 1 hour at room temperature. Following the washing of the membrane, it was treated with luminol and peroxide (Thermo Scientific Pierce ECL; 32106, USA) mixed in a 1:1 ratio and viewed under the UVP Syngene GBox Chemi-XRQ Gel Documentation System (version: 4.03.05 Cambridge, UK). The β-actin antibody was used as a loading control and viewed following washing steps after treatment with a secondary antibody. Densitometry analyses were performed using ImageJ (USA National Institutes of Health, available at: https://imagej.net/ij/). Arbitrary densitometry units were assigned; data were presented as mean±SEM. The expression of the target protein was normalized to that of the β-Actin protein expression. The WB data in the figures and supplemental figures represent more than three independent experiments.

Statistical Analysis

The data obtained from the study were made using the program developed by the faculty members of Inonu University Faculty of Medicine, Department of Biostatistics and Medical Informatics, and made available as an open access program[21]. In data analysis, first of all, controls were made to prevent missing and erroneous data and excessive variable/outlier problems, and corrections were made if necessary. Quantitative data were summarized as mean±SD, and qualitative data were summarized as numbers (percentage). In order to compare the examined variables between groups with a one-way analysis of variance, conformity to normal distribution and homogeneity of variances (Levene's test) were checked. When these assumptions were met, the difference between the group means, one-way analysis of variance, and multiple comparisons were made with the Tukey HSD test when the variances were homogeneous and with the Tamhane T2 when they were not. In cases where the normality assumptions were not met, the Kruskal-Wallis H test was used, and the Conover test was used for multiple comparisons. A $p<0.05$ was considered statistically significant.

Results

Evaluation of Infarct Area

According to our findings, infarct areas were determined with TTC staining in Figure 1. No infarct area was observed in the brains of sham-operated rats. Infarct area increased by 44.25% in the CI group compared to the Sham group, while it was 13.13% in the Asp+CI group treated with Asp, and the decrease of 31.12% in the Asp+CI group was statistically significant ($p<0.05$). In fact, areas were 29.76% in the CI+Asp group, 27.43% in the CI+Asp+3-MA group, and 16.43% in the Asp+CI+3-MA group. Asp application before CI provided a statistically significant decrease in infarct areas compared to the groups in which Asp was applied after CI ($p<0.05$).

Evaluation of the Level of Markers Associated with the Autophagy Pathway

To investigate whether Asp, 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, ATG-5, ATG-7, and p62) by WB analysis in Figure 2A. The concurrent increase in Beclin-1, ATG-5, and ATG-7 proteins and a decrease in p62 indicate activation of autophagy.

Following 60 min of CI, Beclin-1 protein levels were decreased in the CI group. However, Asp treatment after CI increased more than in the CI+Asp groups ($p<0.05$) (Figure 2B). According to our results, the increase in ATG-5 and ATG-7 protein levels in 3-MA treated groups after CI was statistically significant compared to CI groups (Figure 2C-D) ($p<0.05$). Additionally, the p62 protein levels (Figure 2E) were increased after CI and significantly reduced at the end of CI after the administration of 3-MA ($p<0.05$).

Evaluation of the Level of Markers Associated with the Apoptosis Pathway

The cleavage of caspase-3 and 9 plays a critical role in cell death in apoptosis by activating DNA fragmentation, and their activation was assessed by WB (Figure 3A). Following CI, cleaved caspase-3-9 (Figure 3B) protein levels decreased in CI groups. However, Asp treatment increased the cleaved caspase-3-9 (Figure 3C), but administration of 3-MA decreased ($p<0.05$). In addition, Bcl-2 (Figure 3D) and BAX (Figure 3E) protein levels were lower in the CI compared to the Sham group ($p<0.05$). The increase in Bcl-2 and BAX

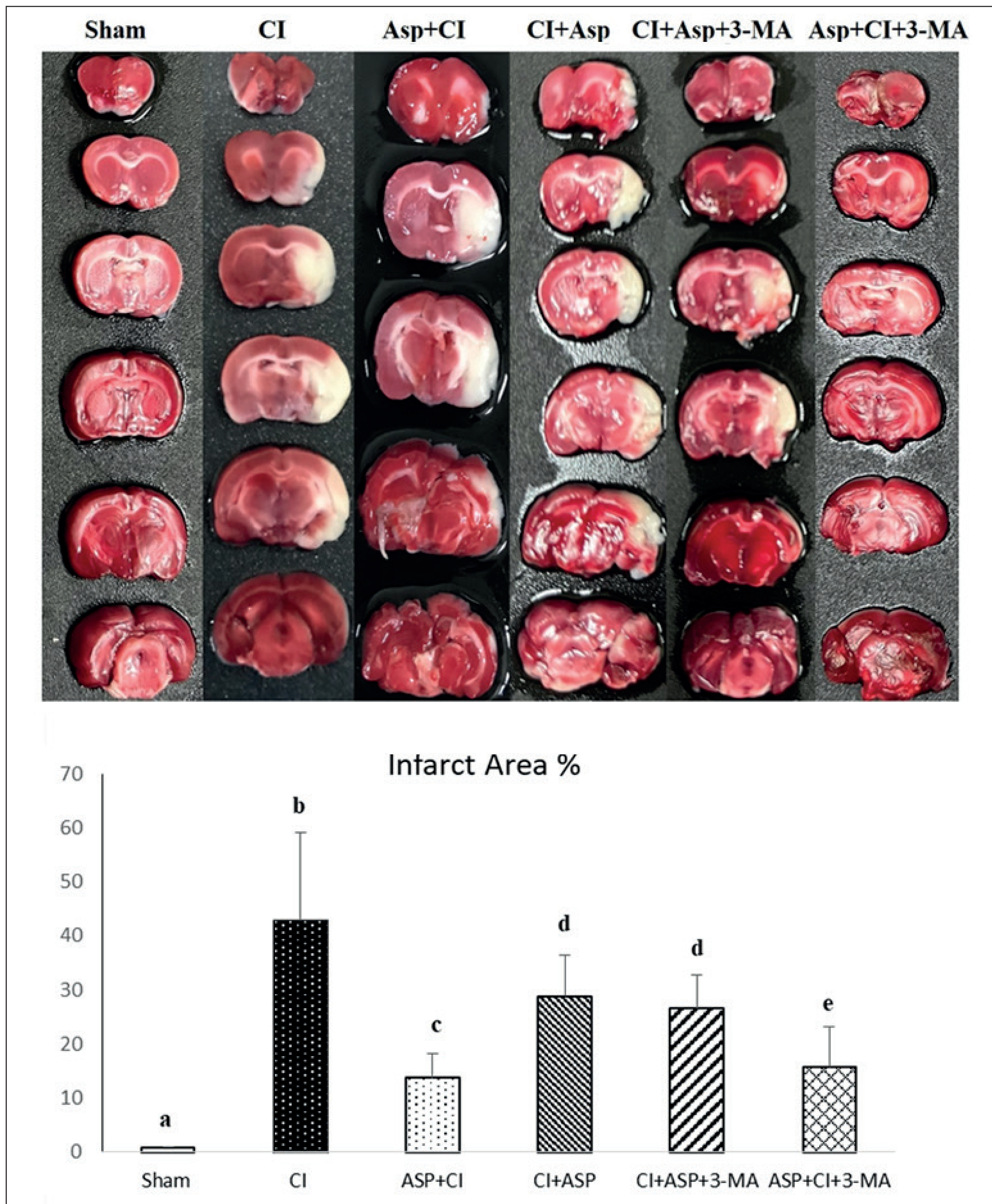

Figure 1. Asp treatments decreased the infarct area after CI. During the reperfusion period after CI, when the duration of Asp treatment was extended from 3 days, it was determined that the infarct area decreased even more. Different letters (a, b, c, d, and e) were statistically different from each other (p<0.05). The Kruskal-Wallis H test was used to evaluate the infarct area results of the groups. When significant differences were detected between groups, multiple pairwise comparisons were made using the Mann-Whitney U test with Bonferroni correction (p<0.05).

protein levels in Asp-treated groups was statistically significant compared to CI group (p<0.05). The results showed that Asp significantly reduced the key apoptotic protein levels in the CI.

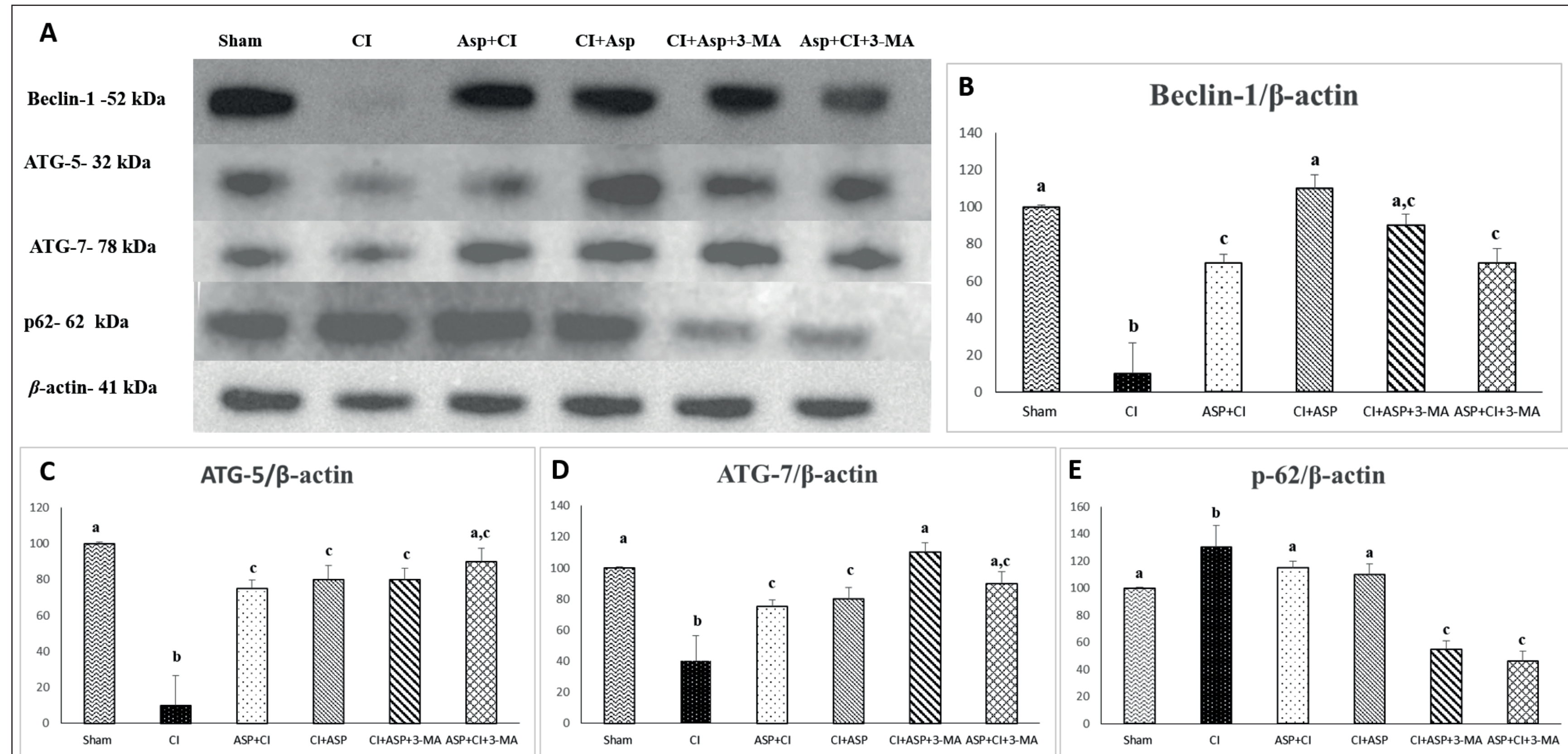

Figure 2. Asp treatment induced autophagy marker protein levels during the reperfusion period after CI. Western images of autophagy markers (**A**), Beclin/β-actin (**B**), ATG-5/β-actin (**C**), ATG-7/β-actin (**D**), p-62/β-actin (**E**). Asp treatment groups marked with different letters (a, b, and c) are statistically different from each other (p<0.05). The differences between the groups marked with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate the Western blot results of the groups. When significant differences were detected between the groups, multiple pairwise comparisons were made using the Mann-Whitney U test with Bonferroni correction.

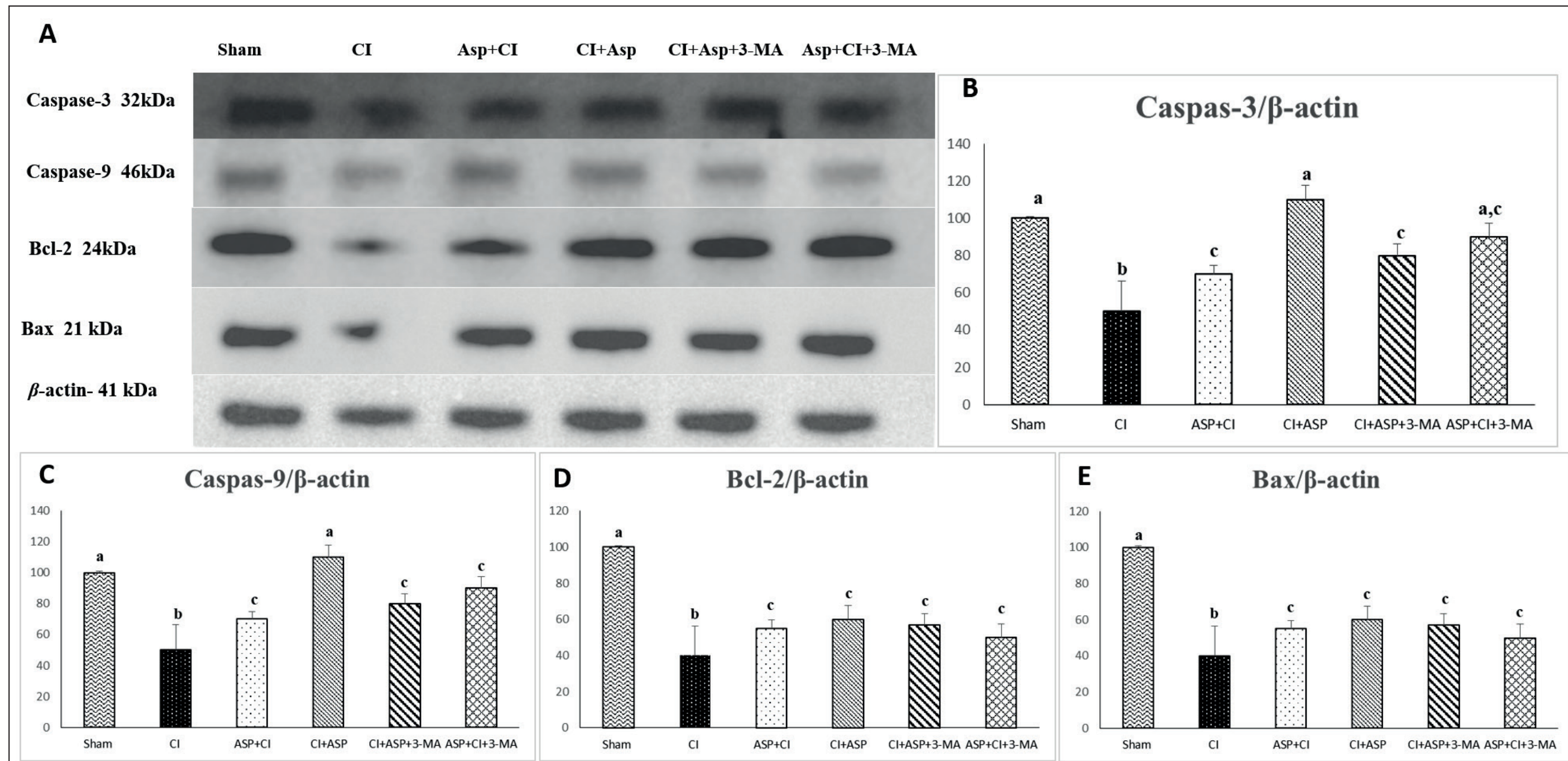

Figure 3. Asp treatment is effective on apoptosis markers suppressed during CI/R. Western images of apoptotic and antiapoptotic markers (**A**), cleaved caspase-3/β-actin (**B**), caspase-9/β-actin (**C**), Bcl-2/β-actin (**D**), Bax/β-actin (**E**). Groups marked with different letters (a, b, and c) are statistically different ($p<0.05$). The differences between the groups marked with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate the Western blot results of the groups. When significant differences were detected between the groups, multiple pairwise comparisons were made using the Mann-Whitney U test with Bonferroni correction.

Discussion

The brain stands as the most susceptible organ to various ailments, including ischemia. Cerebral ischemia or stroke, a leading global cause of mortality and disability, necessitates the swift restoration of blood flow as the primary treatment. Despite the successful reestablishment of cerebral blood flow, ensuring the supply of oxygen and energy, a risk of progressive neuronal degeneration remains. Unfortunately, numerous interventions targeting such pathways have proven[22] only partially effective, with countless clinical trials failing to demonstrate therapeutic efficacy against ischemic stroke. Consequently, there is a growing interest in unraveling the mechanisms underlying endogenous neuronal protection and repair, offering potential avenues for innovative strategies[23].

Asprosin has been reported[24] to have multiple effects on CNS and peripheral tissues and organs. Asprosin has an essential impact on regulating appetite, glucose metabolism, IR, cell apoptosis, and autophagy *via* multiple downstream signaling pathways. However, studies in literature examining behavioral tests following Asp injection in a CI/R model are quite limited[25].

Furthermore, in CI/R studies[26,27], studies in which reperfusion was provided for longer than 24 h and otofagia and apoptosis pathways were examined are very limited. In a study[23] on a 60-min CI model, Asp was administered before the initiation of reperfusion, and it was reported that Asp improved the infarct area score in the neurologic evaluation performed of reperfusion. In CI/R studies performed to date, it has been reported[17] that agents with autophagic activity injection reduce the infarct area after 24 h[27]. In one[28] of the CI/R studies in which reperfusion was provided longer than 24 h, the infarct area was determined on the 3rd day of reperfusion. In addition, it can be said that some reduction in the infarct area can be observed as the reperfusion time is prolonged. However, Asp 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 Asp 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. According to our results, it can be said that after 60 min of ischemia, before and after Asp administration, neuronal cell loss in the penumbra area is prevented.

Some studies[29] indicate that autophagy plays a protective role in CI, while others[30] indicate that autophagy plays a role as a cell death pathway in CI[25]. Asp has been suggested to have an effect on the autophagy pathway in myocardial infarction; however, whether autophagy is a beneficial ischemia process has not been fully clarified in these studies. To date, the impact of Asp on the role of autophagy has not only been studied in CI/R injury but also in neonatal cerebral hypoxic-ischemia models. According to our results, it can be said that 3-day Asp treatment stimulates autophagy, and Asp treatment increases autophagy levels even further. In addition, it was determined that the level of cleaved caspase-3 protein increased during the 3-day reperfusion period, but it decreased over time during the ongoing reperfusion period. However, distinguishing between autophagy and apoptosis as cell death pathways within the initial 24 hours can pose challenges. This is because, according to one study[31], neuronal autophagy was reported to be induced after 48 hours, contrasting with other organs. Autophagy, alongside apoptosis, can function as a cell death pathway when the physiological stress conditions surpass the cell's adaptive capabilities. Therefore, analyzing autophagy within the first 24 hours in CI may portray it as a cell death pathway. The difficulty in determining whether autophagy serves as a cell death or survival pathway in CI may arise from the fact that autophagy is typically examined in the infarct area, which predominantly consists of the core region where cells have already committed to the death pathway. To gain a clearer understanding, it is essential to focus on the penumbra area during autophagy examination, as this region holds the key to cell fate. Optimal autophagic activity in ischemic cells can promote cellular survival by eliminating damaged organelles and cellular components instead of pushing the cell toward demise[32]. Previous research[33] has suggested an intricate interplay between autophagy and apoptosis, indicating the potential sharing of molecular inducers and regulatory mechanisms. Moreover, it has been proposed that autophagy can influence caspase-mediated apoptosis. Our findings align with this perspective, as the levels of Beclin-1, ATG-5, and ATG-7 were observed to increase, while p-62 and cleaved caspase-3 caspase-9 decreased.

Conclusions

Asp has protective and therapeutic effects against CI/R damage. While applying Asp before ischemia activates the autophagy pathway more, applying Asp after ischemia protects the neuronal death/survival balance by activating the apoptosis pathway more.

Conflict of Interest

The authors declare that they have no conflict of interest to disclose.

Ethics Approval

This study was carried out with the approval of the Ethical Committee of Experimental Animals of the Faculty of Medicine at Inonu University (2021/19-3).

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Authors' Contributions

T.K. and S.S. conducted experiments and analyzed the data. T.K.,Y.F., T.S.and T.C. was responsible for the data curation. T.K. wrote the manuscript. S.S. was responsible for the project administration. All authors have read and agreed to the published final version of the manuscript. SS is the guarantor of this work and takes responsibility for the in-tegrity of the data.

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Data Availability

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

References

1) Cimarosti H, Henley JM. Investigating the mechanisms underlying neuronal death in ischemia using in vitro oxygen-glucose deprivation: potential involvement of protein sumoylation. Neuroscientist 2008; 14: 626-636.
2) Tian XH, Chen X, Jiang GF. Effects of comprehensive care measures based on the HAPA model on self-care, neurotransmitters and clinical outcomes in cerebral infarction patients. Eur Rev Med Pharmacol Sci 2023; 27: 4462-4470.
3) Shi LJ, Chagari B, An A, Chen MH, Bao Y, Shi W. Genetic Connection between Hyperglycemia and Carotid Atherosclerosis in Hyperlipidemic Mice. Genes (Basel) 2022; 13: 510-525.
4) Jiang HF, Guo YQ, Rehman FU, Jing L, Zhang JZ. Potential cerebrovascular protective functions of lycium barbarum polysaccharide in alleviating hyperglycemia-aggravated cerebral ischemia/reperfusion injury in hyperglycemic rats. Eur Rev Med Pharmacol Sci 2022; 26: 7379-7394.
5) Wang XX, Zhang B, Xia R, Jia QY. Inflammation, apoptosis and autophagy as critical players in vascular dementia. Eur Rev Med Pharmacol Sci 2020; 24: 9601-9614.
6) Rao XL, Liu LL, Huang J, Chen J. Neuroprotective effects of visnagin on cerebral ischemia-reperfusion injury rats and the underlying mechanisms. Eur Rev Med Pharmacol Sci 2022; 26: 4371-4379.
7) Wong A, Bhuiyan MIH, Rothman J, Drew K, Pourrezaei K, Sun D, Barati Z. Near infrared spectroscopy detection of hemispheric cerebral ischemia following middle cerebral artery occlusion in rats. Neurochem Int 2023; 162: 105-109.
8) Yao X, Kong X, Ren J, Cui Y, Chen S, Cheng J, Gao J, Sun J, Xu X, Hu W, Li H, Che F, Wan Q. Transcranial direct-current stimulation confers neuroprotection by regulating isoleucine-dependent signalling after rat cerebral ischemia-reperfusion injury. Eur J Neurosci 2023; 58: 3330-3346.
9) Yilmaz B, Terekeci H, Sandal S, Kelestimur F. Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. Rev Endocr Metab Disord 2020; 21: 127-147.
10) Tanbek K, Ozerol E, Yilmaz U, Yilmaz N, Gul M, Colak C. Alpha lipoic acid decreases neuronal damage on brain tissue of STZ-induced diabetic rats. Physiol Behav 2022; 248: 113-121.
11) Romere C, Duerrschmid C, Bournat J, Constable P, Jain M, Xia F, Saha PK, Del Solar M, Zhu B, York B, Sarkar P, Rendon DA, Gaber MW, LeMaire SA, Coselli JS, Milewicz DM, Sutton VR, Butte NF, Moore DD, Chopra AR. Asprosin, a Fasting-Induced Glucogenic Protein Hormone. Cell 2016; 21: 566-579.
12) Beutler LR, Knight ZA. Share A Spotlight on Appetite. Neuron 2018; 21: 739-741.
13) Wang CY, Lin TA, Liu KH, Liao CH, Liu YY, Wu VC, Wen MS, Yeh TS. Serum asprosin levels and bariatric surgery outcomes in obese adults. Int J Obes 2019; 43: 1019-1025.
14) Chen S, Wang X, Qiu CM, Hou JN, Wei XY, Xiang CX, Tang MY, Zhang R, Pei HF. Study of the Role and Mechanism of Asprosin/Spartin Pathway in Cardiac Microvascular Endothelial Injury Induced by Diabete Mellitus. Sichuan Da Xue Xue Bao Yi Xue Ban 2019; 50: 827-834.

15) Yu F, Tong LJ, Cai DS. Sevoflurane inhibits neuronal apoptosis and expressions of HIF-1 and HSP70 in brain tissues of rats with cerebral ischemia/reperfusion injury. Eur Rev Med Pharmacol Sci 2020; 24: 5082-5090.
16) Metab ESJJCE. Guidelines for the care and use of experimental animals. 1997; 82: 40A.
17) Yilmaz U, Tanbek K, Gul S, Gul M, Koc A, Sandal S. Melatonin Attenuates Cerebral Ischemia/Reperfusion Injury through Inducing Autophagy. Neuroendocrinology 2023; 10: 1035-1050.
18) Romere C, Duerrschmid C, Bournat J, Constable P, Jain M, Xia F, Saha PK, Del Solar M, Zhu B, York B, Sarkar P, Rendon DA, Gaber MW, LeMaire SA, Coselli JS, Milewicz DM, Sutton VR, Butte NF, Moore DD, Chopra AR. Asprosin, a Fasting-Induced Glucogenic Protein Hormone. Cell 2016; 165: 566-579.
19) Chaurasia M, Gupta S, Das A, Dwarakanath B S, Simonsen A, Sharma K. Radiation induces EIF2AK3/PERK and ERN1/IRE1 mediated pro-survival autophagy. Autophagy 2019; 15: 1391-1406.
20) Tanbek K, Yilmaz U, Gul M, Koc A, Sandal S. Effects of central FGF21 infusion on the glucose homeostasis in rats (brain-pancreas axis). Arch Physiol Biochem 2023; 1-8.
21) Arslan AK, Yasar S, Colak C, Yologlu S. WSSPAS: Web-Based Sample Size & Power Analysis Software. Turkiye Klinikleri Journal of Biostatistics 2018; 3: 1-34.
22) Stankowski JN, Gupta R. Therapeutic targets for neuroprotection in acute ischemic stroke: lost in translation? Antioxid Redox Signal 2011; 10: 1841-1851.
23) Koizumi S, Hirayama Y, Morizawa YM. New roles of reactive astrocytes in the brain; an organizer of cerebral ischemia. Neurochem Int 2018; 119: 107-114.
24) Albayrak S, Aydin MA, Ugur K, Hanbeyoglu O, Aydin S, Erol E, Kilinc A, Acar V, Sahin I, Aydin S. Subfatin, asprosin, alamandine and maresin-1 in cerebral ischemia, intracranial and subarachnoid hemorrhages. Eur Rev Med Pharmacol Sci 2023; 27: 4471-4480.
25) Farrag M, Ait Eldjoudi D, González-Rodríguez M, Cordero-Barreal A, Ruiz-Fernández C, Capuozzo M, González-Gay MA, Mera A, Lago F, Soffar A, Essawy A, Pino J, Farrag Y, Gualillo O. Asprosin in health and disease, a new glucose sensor with central and peripheral metabolic effects. Front Endocrinol 2023; 13: 101-119.
26) Shi L, Liang F, Zheng J, Zhou K, Chen S, Yu J. Melatonin regulates apoptosis and autophagy via ROS-MST1 pathway in subarachnoid hemorrhage. Front Mol Neurosci 2018; 26: 11-93.
27) Zheng Y, Hou J, Liu J, Yao M, Li L, Zhang B. Inhibition of autophagy contributes to melatonin-mediated neuroprotection against transient focal cerebral ischemia in rats. J Pharmacol Sci 2014; 3: 354-364.
28) Liu ZJ, Ran YY, Qie SY, Gong WJ, Gao FH, Ding ZT. Melatonin protects against ischemic stroke by modulating microglia/macrophage polarization toward anti-inflammatory phenotype through STAT3 pathway. CNS Neurosci Ther 2019; 25: 1353-1362.
29) Zhao Y, Zhang X, Chen X, Wei Y. Neuronal injuries in cerebral infarction and ischemic stroke: From mechanisms to treatment (Review). Int J Mol Med 2022; 49: 15-24.
30) Wu M, Zhang H, Kai J, Zhu F, Dong J, Xu Z. Rapamycin prevents cerebral stroke by modulating apoptosis and autophagy in penumbra in rats. Ann Clin Transl Neurol 2018; 5: 138-146.
31) Alirezaei M, Kemball CC, Flynn CT, Wood MR, Whitton JL, Kiosses WB. Short-term fasting induces profound neuronal autophagy. Autophagy 2010; 6: 702-710.
32) Carloni S, Riparini G, Buonocore G, Balduini W. Rapid modulation of the silent information regulator 1 by melatonin after hypoxia-ischemia in the neonatal rat brain. J Pineal Res 2017; 63: 24-34.
33) Grishchuk Y, Ginet V, Truttmann AC, Clarke PG, Puyal J. Beclin 1-independent autophagy contributes to apoptosis in cortical neurons. Autophagy 2011; 7: 1115-1131.