# Effect of Biochanin-A on the Cerebellum in Cerebral Ischemia Reperfusion Injury

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## Abstract

**Aim:** To examine the protective effect of Biochanin A (BCA) on the cerebellum in cerebral ischemia reperfusion injury.

**Methods**: 24 Wistar albino female rats were divided into 3 groups. Control, Ischemia reperfusion (IR), IR +BCA (20 mg/kg intraperitoneal) group. A micro bulldog clamp was placed on the left common carotid artery of the rats and cerebral ischemia was applied for 2 hours. After cerebral ischemia, the clamp was removed and reperfusion was performed for 24 hours. After 7 days, all rats were decapitated and the protective effects of BCAon the cerebellum were evaluated by immunohistochemically analyses.

**Results:** In the IR group; S100 expression was also observed positive in neurons and neuroglia in the pia mater, molecular layer, Purkinje cells in the ganglionic layer and granule cells in the granular layer, and neuroglia in the white matter. In the IR+ BCA group, it was observed that the immune activity in the granule cells and Purkinje cells in the granular layer was reduced compared to the IR group.

**Conclusions**: We suggest that BCA treatment has a potential therapeutic role in alleviating inflammation in the cerebellum after cerebral ischemia reperfusion.

***Keywords:*** *Cerebral ischemia, Biochanin A, S100, cerebellum*

## 1. Introduction

Stroke is the second cause of death and a major cause of disability worldwide[1]. It is examined in two types: ischemic stroke and hemorrhagic stroke. Hemorrhagic stroke is less common[2]. Ischemic stroke is a condition in which the cerebral artery is suddenly blocked by a thrombus and the brain remains hypoxic. Reperfusion is the restoration of blood flow to the ischemic area. This situation increases oxidative stress and inflammation, causing increased neuronal damage. This is called cerebral ischemia/reperfusion injury[3].

Stroke treatments include recanalization and neuroprotection[4] . Today, functional disorders are significantly reduced by using thrombolytic agents and intravascular techniques. Restoration of blood flow and oxygenation often leads to exacerbation of cerebral tissue damage and deepening of the inflammatory response[5].Cerebral ischemia/reperfusion (I/R) is a serious condition that causes cerebral edema, cerebral hemorrhage, and neuronal death. Mechanisms causing cerebral ischemia/reperfusion injury (I/R); Increase in intracellular Ca2+ level, production of free oxygen radicals, increase in pro-inflammatory cytokines, inflammation and apoptosis[6]. Recently, data from many studies have supported the idea that I/R triggers inflammatory cascades in the brain that can further increase tissue damage[6,7]. Upregulation of proinflammatory cytokines occurs within minutes of occlusion. Therefore, effectively preventing and controlling cerebral I/R is of great clinical value [8]. Many structural and biochemical changes can be observed in rats exposed to cerebral ischemia reperfusion, not only in the brain but also in regions far from the ischemic focus. The cerebellum is one of these areas. Apoptosis and inflammation indicate the effects on the cerebellum[9,10].

Phytoestrogens, whose chemical structure is similar to mammalian estrogen, selectively bind to estrogen receptors (ERs) exert estrogenic , anti-estrogenic effects[11,12]. Phytoestrogens are known to be useful in various neurodegenerative disorders such as acute ischemic stroke[13].

BCA (C16H1205) is a phytoestrogen isolated from red clover, chickpeas or other legumes and is an O-methylated natural isoflavonoid. The studie has shown that BCA reduces inflammatory mediators and has a cytoprotective effect[12]. In addition, it is thought that BCA produces neuroprotective effects by inhibiting the inflammatory response in rats subjected to cerebral ischemia/reperfusion, especially as a result of the suppression of p38 signaling pathways[14]. BCA, one of the promising agents in the treatment of

*Corresponding Author: Öner Avınca, droneravinca@gmail.com,* *Received: 25.01.2024, Accepted: 06.03.2024, Available Online Date: 11.03.2024 Cite this article as: Avinca Ö, Kaplan Ö, Aşır F. Effect of Biochanin-A on the Cerebellum in Cerebral Ischemia Reperfusion Injury. J Cukurova Anesth Surg. 2024; 7(1): 47-51. https://doi.org/10.36516/jocass.1425526* 

ischemic stroke patients, needs further investigation.

In our study; we aimed to investigate the protective effects of BCA on the cerebellum in cases of experimental cerebral ischemia reperfusion damage.

## 2. Materials and methods

### 2.1 Animals

Our study received ethics number 646615 and 2024/01 date by Dicle University animal experiments local ethics committee. After the rats were divided into 3 equal groups (n:8), general anesthesia was administered with the rats using 90 mg/kg ketamine hydrochloride and 10 mg/kg xylazine (intramuscular) after a 6-hour fast before the operation

The rats were fixed and the neckline was cleaned with povidone-iodine. Common carotid artery was reached. A micro bulldog clamp was placed approximately 1 cm proximal to the left common carotid bifurcation, common carotid artery occlusion was performed, and cerebral ischemia was applied for 2 hours. After cerebral ischemia, the clamp was removed and the tissues were placed in their old anatomical location. The skin and subcutaneous fascia were sutured and the cerebral tissues were reperfused for 24 hours.

### 2.2 Experimental Groups

1. Control group: Cerebral artery occlusion will not be performed. Only the left common carotid artery will be isolated and the tissues will be closed again. Animals will be given 1 cc of DMSO intraperitoneal for 7 days.

2. Ischemia-Reperfusion (IR) group: Cerebral IR procedure will be applied. Animals were given 1 cc of DMSO intraperitoneal for 7 days.

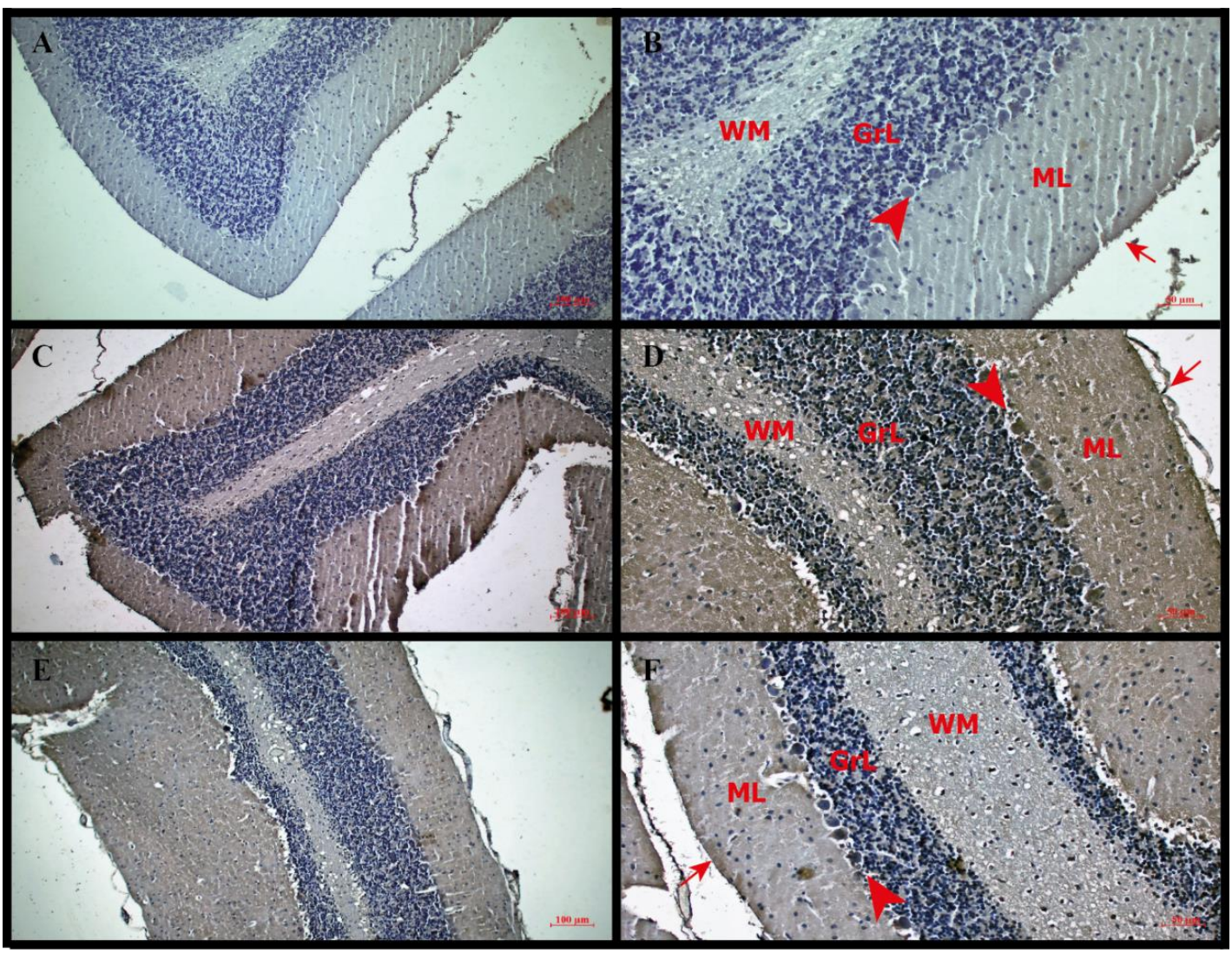
3. IR +BCA group: After the IR procedure, 20 mg/kg BCA was given to the animals intraperitoneal for 7 days. After the experimental protocol was completed (at the end of the 7th day), the animals were sacrificed by drawing blood from the heart under general anesthesia. Cerebellar tissue of rats was removed.

### 2.3 Histological Tissue Protocol

The collected cerebellum tissue was fixed in chain formalin and subjected to routine histological follow-up. After fixation, alcohol series, xylene and paraffin processes, the tissues were embedded in paraffin blocks. Paraffin blocks were cut to 5 microns using a microtome and a slide was taken. Immunohistochemically staining was performed by applying S100 antibody to the sections.

Figure 1

S100 immune staining of cerebellum tissue sections



### 2.4 Immunohistochemically staining

Sections of the cerebellum were dewaxed, passed through an alcohol series, and washed with distilled water. 3% hydrogen peroxide (H2O2) was used to block endogenous peroxidase activity. The sections were washed in PBS and then immersed in blocking solution. Before washing, the solution was decanted and the sections were incubated with primary antibody S100 (catalog no: Santa Cruz Biotechnology Inc. CA, USA) overnight at +4°C. Sections were biotinylated and then reacted with streptavidin peroxidase for 15 min. Diaminobenzidine (DAB) chromogen was used as a chromogen to observe the color change after PBS washing. Reactions were stopped with PBS solution and sections were counterstained with hematoxylin dye. Slides were viewed with a Zeiss Imager A2 light microphone. All images were processed and quantified using Image software[15].

### 2.5 Images Analysis

The staining intensity of S100 expression was measured by Image J software (version 1.53, http://imagej. nih.gov/ij). Measurement was calculated by the method of Crowe et al[16].Quantification was recorded by analyzing ten fields from each specimen per group[17]. In specimens, the brown color stands for the positive expression of the antibody of interest while the blue color represents a negative expression of the antibody of interest. Signal intensity (expression) from a field was calculated by dividing the intensity of the antibody of interest by the whole area of the specimen. A value for staining area/whole area was calculated for each specimen from ten fields. An average value was measured for groups and analyzed for semi-quantitative immunohistochemistry scoring.

### 2.6 Statistical Analysis

IBM SPSS 25.0 software (IBM, Armonk, New York, USA) was used for statistical analysis. The data were recorded as median (quartile 25 and quartile 75). Shapiro-Wilk test was used to evaluate statistical distribution. Multiple group comparisons were done with the Kruskal wallis and posthoc Dunn's test. Significance was considered for p-values<0.05.

**Figure 2**

Negative Control of S100 immune staining of cerebellar sections. A: 100 µm, magnification: 10X; B: 50 µm, magnification: 20X

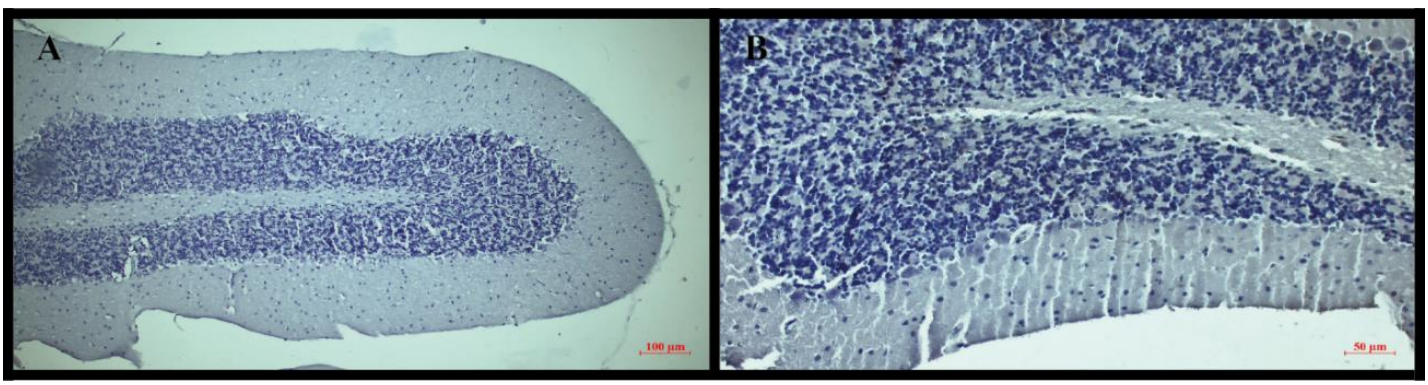



**Figure 3**

Graphical illustration of signal intensity

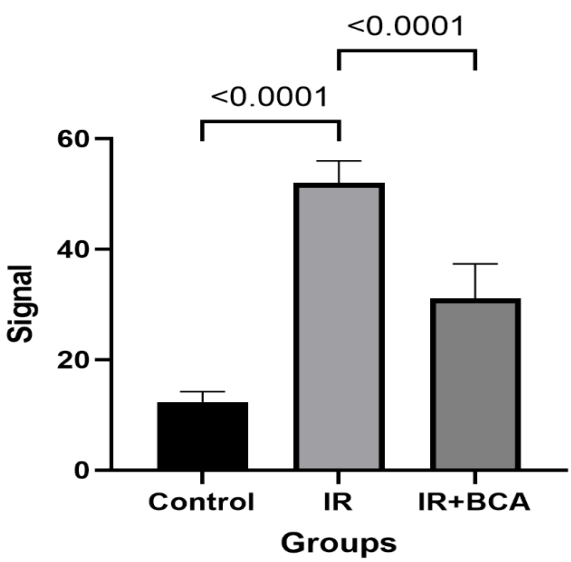



**Table 1**

S100 signal level in groups

| | Control | IR | IR+BCA |
|---|---|---|---|
| | Median (Q1-Q3) | Median (Q1-Q3) | Median (Q1-Q3) |
| S100 signal | 12 (11-14) | 48 (52-55)* | 32 (25-37)** |

## 3. Results

### 3.1 Immunohistochemically Findings

S100 immune staining of cerebellum tissue sections is shown in Figure 1. In the images of the control group, S100 expression was observed to be negative in the white matter, granular layer, ganglionic layer and molecular layer (Figure 1a and 1b). S100 expression generally increased due to inflammation after cerebral ischemia reperfusion (IR). Increased S100 expression was observed in neurons and neuroglia in the pia mater, molecular layer, Purkinje cells in the ganglionic layer, and granule cells in the granular layer, compared to the control group. S100 expression was also observed positive in neuroglia in the white matter (Figure 1c and 1d). After BCA treatment, S100 expression decreased in the cerebellum tissue due to the anti-inflammatory properties of BCA. It was observed that immune activity, especially in the granule cells and Purkinje cells in the

granular layer, decreased compared to the IR group (Figure 1e and 1f).

Figure 1. Cerebellar sections of control (a and b), IR (c and d) and IR+BCA (e and F) groups. WM: white matter, GrL: granular layer, ML: molecular layer, arrow: pia-mater, arrowhead: Ganglionic layer (Purkinje cells). A, C and E: 100 µm, magnification: 10X; B, D and F: 50 µm, magnification: 20X,

Figure 2. negative Control of S100 immune staining of cerebellar sections. A: 100 µm, magnification: 10X; B: 50 µm, magnification: 20X

### 3.2 Statistical Findings:

Semiquantitative measurement of S100 immune staining was shown in Table 1. Graphical illustration of signal intensity was shown in Figure 3 with significance.

## 4. Discussion

Ischemia (I) is the hypoxic effect that occurs when there is no or little blood flow in tissues and organs. Reperfusion (R) is the resumption of blood flow. Both situations are dangerous. The harmful effects of free oxygen radicals (ROS) formed as a result of ischemia increase with the reperfusion into the tissues during the reperfusion phase.[18]. The brain is one of the most important organs to be affected by ischemia reperfusion. In cerebral ischemia/reperfusion (I/R) injury, serious conditions such as brain edema, brain hemorrhage and neuron death may occur[14].

Structural and biochemical changes can also be observed of outside ischemic area in animals exposed to cerebral ischemia[19]. The studie has shown that the cerebellum is one of the affected areas. Edema occurring in the brain after ischemia in animals compresses the cells in the contralateral hemisphere. Increased intracellular calcium indirectly causes metabolic stress. As a result, various inflammatory mediators, growth factors and heat shock proteins are induced[19].

S100 proteins are known to be a family of low-molecular-weight calcium-binding proteins found in vertebrates. S100 B protein, which belongs to th5-3 e S100 family, is released by astrocytes in the brain[20]. Trophic or apoptotic depending on the level of secretion leads to results. This toxic effect of S100 is based on its ability to induce pro-inflammatory cytokines, oxidative stress enzymes, especially iNOS 31-35[21]. Sun at all. showed that proteins belonging to the S100 family were upregulated in the brains of mice in which focal cerebral ischemia caused reperfusion[22].

In our study, we chose the S100 antibody to examine the inflammation in the cerebellum tissue of rats in which we created cerebral ischemia reperfusion. That S100 expression was negative in the white matter, granular layer, ganglionic layer and molecular layer in the images of the control group (Figures 1a and 1b), S100 expression generally increased due to inflammation after cerebral ischemia reperfusion (IR). Increased S100 expression was observed in neurons and neuroglia in the pia mater, molecular layer, Purkinje cells in the ganglionic layer, and granule cells in the granular layer, compared to the control group. We also observed positive S100 expression in neuroglia in the white matter (Figures 1c and 1d).

BCA is a type of phytoestrogen that shows anti-tumorigenesis, anti-oxidation, anti-inflammatory and hypoglycemic effects. Recently, various studies have been conducted to examine the effects of BCA on cerebral ischemia reperfusion. In a study, BCA was used to inhibit the inflammatory response in rats with cerebral ischemia/reperfusion injury, and BCA showed a neuroprotective effect by suppressing inflammation[14]. BCA reduced cerebral ischemia reperfusion injury by reducing leukocyte migration [23].

In the study, in the cerebellum of rats in the ischemia reperfusion + BCA group; We observed that immune activity in granule cells and Purkinje cells in the granular layer was reduced compared to the IR group (Figures 1e and 1f).

## 5. Conclusion

The results indicate that BCA treatment exerts anti-inflammatory effects in cerebellum tissue following cerebral ischemia-reperfusion. The observed decrease in S100 expression, especially in key cell types, suggests a potential therapeutic role for BCA in mitigating inflammation associated with cerebral IR injury. Further studies and analyses may be needed to explore the underlying mechanisms and to validate the potential clinical relevance of these findings

## Statement of ethics

Our study received ethics number 646615 and 2024/01 date by Dicle University animal experiments local ethics committee.

## Conflict of interest statement

The authors declare that they have no financial conflict of interest with regard to the content of this report.

## Funding source

The authors received no financial support for the research, authorship, and/or publication of this article.

## Author Contributions

All authors contributed equally to the article. All authors read and approved the final manuscript.

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