Chlorogenic Acid Protects Cell Death in the Cerebellum through Anti-apoptotic Protein Bcl2 in Transient Global Ischemia Cases

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Background: Cerebellum is one of the vital components of the brain that will be affected by ischemia-reperfusion (IR) injury. IR injury will increase free radicals, which in turn can trigger apoptosis and cell death. Therefore, this study was conducted to examine the effect of chlorogenic acid administration on apoptosis and the number of cells in the cerebellum of rats with global ischemic transients.

Materials and methods: Wistar rats were divided into five groups: sham-operated (C1), IR (C2), IR + 15 mg/kgBW chlorogenic acid (T1), IR + 30 mg/kgBW chlorogenic acid (T2), and IR + 60 mg/kgBW chlorogenic acid (T3). C2, T1, T2, and T3 groups received bilateral common carotid occlusion (BCCO) surgery to induce IR injury. Thirty minutes after BCCO surgery, T1, T2, and T3 rats were administered chlorogenic acid in various doses intraperitoneally. RNA extraction and real-time polymerase chain reaction (PCR) measurements were then performed on NeuN, Bcl2, Bax, caspase 3, as well as on GAPDH as housekeeping genes.

Results: There were significant differences in NeuN expressions between groups, with the highest expression shown in C1 followed by T3. Bcl2 expressions were also significantly different between groups, and rats in C1 and T3 showed to be significantly higher compared to C2, while T1 was significantly lower than C1. However, Bax and caspase 3 expressions showed no significant differences.

Conclusion: Chlorogenic acid in 60 mg/kgBW dose increases NeuN expression and Bcl2 mRNA expression after transient global ischemia. These increases might correlate with the heightened level of protection against apoptosis in the cerebellum, hence showing its potential in protecting neuron cells.

Keywords: transient global ischemia, chlorogenic acid, cerebellum, apoptosis

Introduction

The transient global ischemia model is an experimental animal model to simulate cases of global ischemia in humans, such as cases of heart failure and shock. Complications of cardiovascular disease is one of many effects that is caused by the occurrence of transient global ischemia conditions in the brain. In addition to cardiovascular cases, ischemic
strokes are also often occurred following ischemia condition. Ischemic stroke is more common than hemorrhagic stroke, with a prevalence of 67.1% of all stroke cases. Ischemic stroke is one of the most common causes of death or results in disabilities, including cognitive function. The prevalence of post-stroke cognitive impairment is 20-80%. Brain is known to be very sensitive to ischemia conditions.

Ischemia-reperfusion (IR) injury is a condition where blood flow is restored after ischemia. This condition causes an increase in free radicals, resulting in oxidative stress. One area of the brain that is affected by IR injury is the cerebellum. The cerebellum plays a pivotal role in regulating the sequence of motor activity and its speed, as well as controlling smooth movement from one muscle movement to the next. The cerebellum also helps regulate the intensity of muscle contractions when there is a change in the load on the muscles by controlling the contractions of agonist and antagonist muscle groups so that they can adjust to the motor signals generated by the motor cortex and other parts of the brain.

Cerebellum function can be disrupted as a result of IR injury due to apoptosis resulting in cell death. Oxidative stress is the mechanism underlying the occurrence of neuron damage under ischemia conditions, due to increased production of free radicals under ischemia and reperfusion conditions.

Oxidative stress can be overcome by administering polyphenols. Polyphenols themselves consist of a group of phenolic acids, flavonoids, stilbenes, tannins, and diferuloylmethane. One of the compounds included in phenolic acids is chlorogenic acid. Chlorogenic acid is the main phenolic compound found in coffee and has an antioxidant effect. Previous study showed that chlorogenic acid protected hippocampal pyramidal cells by increasing mRNA expression of Bcl2. This study was conducted to examine the effect of chlorogenic acid administration on apoptosis and the number of cells in the cerebellum in rats experiencing transient global ischemia.

**Materials and methods**

**The Construction of Animal Model and Experimental Treatment**

This study was conducted at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, and the Faculty of Medicine, Universitas Tanjungpura, Pontianak. Thirty-five male Wistar rats weighed 200-350 g and aged 3-5 months were used as the animal models in this study. The rats were randomly allocated into five distinct groups: sham operated group (C1), IR group (C2), IR + 15 mg/kgBW chlorogenic acid group (T1), IR + 30 mg/kgBW chlorogenic acid group (T2), and IR + 60 mg/kgBW chlorogenic acid group (T3). C2, T1, T2, and T3 groups received bilateral common carotid occlusion (BCCO) surgery to induce IR injury. Thirty minutes after BCCO surgery, rats in T1, T2, and T3 were injected with chlorogenic acid in various doses. After the treatments, the rats were kept in cages under the natural light-dark cycle. Rats were fed *ad libitum* and had access to food pellets and water throughout the study process. The room temperature was kept between 26-31°C and the humidity was kept in the range of 70-90%.

The construction of an animal model with BCCO method was conducted in reference to previous research protocol. During the surgery process, the rats were anesthetized with an injection of 100 mg/kgBW ketamine (PT Guardian Pharmatama, Jakarta, Indonesia) by intramuscular route. The anterior part of the neck was incised through dulled dissection of the neck muscle and salivary gland to expose the trachea and common carotid arteries. The vagal nerve was then detached from the artery, and a pair of non-traumatic vascular clamp (Dieffenbach Bulldog Clamp, World Precision Instruments, Sarasota, FL, USA) was used to clamp the carotid arteries. The arterial clamping process was carried out for 20 minutes to induce transient global ischemia. The same process was conducted on animals of the sham operated (C1) group except the clamping process. For the treatment groups (T1, T2, and T3), 30 minutes after BCCO surgery, chlorogenic acid (Cat. #C3878-1G, Sigma-Aldrich, St. Louis, MO, USA) was administered to the rats by intraperitoneal injection. Ten mg/mL phosphate buffer saline (PBS) solution was used to dissolve chlorogenic acid. Meanwhile, for the control groups (C1 and C2) received injections containing only PBS solution to ensure that the changes that would occur in the treatment groups were the effects of chlorogenic acid and not due to solvents.

The recovery phase lasted for two days, and the rats were rested in the cage before the spatial memory test was carried out. During the recovery phase, the animals were observed and kept clean in their cages to prevent infection. All the study protocols have been approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Tanjungpura (No. 4931/UN22.9/PG/2022).
**Tissue Preparation**

Rats were sacrificed by cervical dislocation and decapitation on the 10th day after the BCCO surgery and after the spatial memory test was performed. Previously, the rats were anesthetized using chloroform (Cat. #1024451000, Merck, Darmstadt, Germany) by inhalation. Thereupon, the brain was taken, and the cerebellum was isolated and placed in a 1.5 mL microcentrifuge tube containing RNAlater® Stabilization Solution (Cat. #AM7021, Thermo Fisher Scientific, Waltham, MA, USA) and stored in a -80°C freezer.

**Extraction of Cerebellum RNA and Quantification of mRNA Expressions**

The 30 mg of cerebellum was extracted based on a protocol as described in the manual of Favorgen Tissue Total RNA Mini Kit (Cat. #FATRK 001-1, Favorgen Biotech Corp, Ping Tung, Taiwan). Qubit 4 fluorometer (Thermo Fisher Scientific) with Qubit RNA HS Assay Kit (Cat. #Q32852, Thermo Fisher Scientific) was used to quantify total RNA. Afterwards, cDNA was synthesized using 1000 ng of RNA in accordance with manufacturer’s instruction of ExcelRT™ Reverse Transcription Kit II (Cat. #RP1400, Smobio, Hsinchu City, Taiwan) with random primer (total volume in a 20 μL). The cDNA was then stored in the -20°C freezer.

The cDNA amplification was done by polymerase chain reaction (PCR) method using QuantStudio™ 5 DX Real-Time PCR System (Thermo Fisher Scientific). A total volume of 20 μL ExcelTaq™ 2X Fast Q-PCR Master Mix (SYBR, no ROX) (Cat. #TQ1200, Smobio) was used. Five target genes were analyzed: GAPDH as housekeeping genes, NeuN gene as marker for the number of neurons, as well as Bcl2, Bax and caspase 3 genes as markers of apoptosis process. The initial denaturation stage was done in 94°C for 2 minutes. Table 1 contained the different primer sequences, number of cycles, temperature and denaturation, annealing and extension time for each gene. The final extension stage was done for 10 minutes at 72°C. The 2-ΔΔCT method was used to determine the relative expression of the mRNA.

**Statistical Analyses**

Initially, Shapiro-Wilk tests and Levene tests were used to analyze the normality and homogeneity of distribution of the data. To examine the differences between groups, One-Way ANOVA was performed for normally distributed and homogeneous data, alternatively, the Kruskal-Wallis test was performed. As a follow up, post hoc analysis was performed using the LSD or Mann-Whitney test. A \( p < 0.05 \) was considered significant.

**Results**

**Chlorogenic Acid Increases NeuN Expression**

The results of NeuN mRNA expression as a marker of cell number in the cerebellum showed that the C1 group had the highest expression of NeuN (0.99), followed by the T3 group (0.51). Meanwhile, the C2 group had the lowest NeuN expression (0.23) (Figure 1).

Since the normality test showed that the data were not normally distributed, so non-parametric Kruskal-Wallis test was performed and showed significant differences between the 5 groups \( (p=0.001) \). After post hoc using the Mann Whitney test, it was found that there were significant differences between the C1 and C2 groups \( (p=0.001) \), T1 \( (p=0.001) \) and T2 \( (p=0.007) \), as well as between T3 and C2 \( (p=0.007) \) and P1 \( (p=0.001) \).

**Chlorogenic Acid Increases Bcl2 Expression**

Figure 2 showed Bcl2 expression in the cerebellum. It appeared that the C1 group had the highest Bcl2 expression (1.66) compared to the other groups. Meanwhile, the C2 group had the lowest NeuN expression (0.23) (Figure 1).

Since the normality test showed that the data were not distributed normally, Kruskal-Wallis non-parametric

<table>
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<th>Table 1. Primers list and PCR conditions.</th>
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<td><strong>Gene</strong></td>
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<td>Caspase 3</td>
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test was performed to examine the differences between the 5 groups, significant differences were obtained ($p=0.016$). Post-hoc using the Mann-Whitney test was also conducted and significant differences were found between the C2 group and the C1 group ($p=0.002$) and T3 ($p=0.038$), and between the C1 group and T1 group ($p=0.005$).

**Chlorogenic Acid Had No Effects on Bax and Caspase 3 Expressions**

The expression of Bax was shown in Figure 3 where it appeared that the highest expression of Bax was found in the C1 group (1.56). Meanwhile, the lowest Bax expression was found in the T1 group (0.91). However, there were no statistically significant differences between 5 groups after the Kruskal-Wallis test ($p=0.405$).

Meanwhile, the caspase 3 expression of the 5 groups was shown in Figure 4, where it appeared that the C1 group had the highest expression (0.84). Since the data were normally distributed, we performed one-way ANOVA test and found that there was no significant differences between the 5 groups ($p=0.116$).

**Discussion**

In this study, there was an increase in the expression of a neuronal marker, namely NeuN, after administration of chlorogenic acid in transient global ischemia. The mechanism of action of chlorogenic acid is by increasing the expression of Bcl2 mRNA as an anti-apoptotic protein.

IR injury is induced by causing global ischemia for 20 minutes which is done by the BCCO method. Return of blood flow after BCCO will impair IR injury. The oxygen paradox mentions that re-exposure to oxygen which will trigger the production of reactive oxygen species (ROS).14
IR injury will cause various manifestations that will lead to cell death.\textsuperscript{14-16}

Cell death is marked by decreased expression of NeuN as a marker for neuron cells. Cerebellar degeneration as a consequence of transient brain ischemia was measured using neuronal marker NeuN. It is proven that decreased expression of NeuN (+) protein positively correlates with the presence of neuronal death.\textsuperscript{17} Previous research stated that IR injury had an impact on increasing free radical levels, which results in causing apoptotic cell death. In this study, the expression of Bcl2 was lower in the ischemic group compared to the control group and the group that was given chlorogenic acid at a dose of 60 mg/kg BW. Thereupon, there was an increase in the apoptotic signal that caused cell death in the ischemia group that was not given chlorogenic acid.

Chlorogenic acid belongs to a class of polyphenols that have a phenol structure. This structure can donate hydrogen atoms to then convert free radicals into their normal structure. There have been many studies that state the efficacy of chlorogenic acid as an antioxidant, anti-inflammatory, and anti-cancer.\textsuperscript{18}

This study examines the cell death pathway through the mRNA expression of Bcl2, Bax and caspase 3. Real-time PCR examination showed that chlorogenic acid could protect neuronal cells in the cerebellum by significantly increasing Bcl2 mRNA expression as an anti-apoptotic protein. The increase in Bcl2 that occurred was in accordance with several other research on neuronal cell cultures, mesenchymal stem cells and liver tissue which stated that after administration of chlorogenic acid, Bcl2 expression would increase.\textsuperscript{19-23} Bcl2 protects cells by inhibiting shifts in mitochondrial
permeability, binding to pro-apoptotic proteins, reducing the rate of formation of free radicals, reducing the influx of Ca\textsuperscript{2+} to modulate endoplasmic reticulum (ER) stress-induced apoptosis, regulating intracellular pH.\textsuperscript{24}

Bax also regulates apoptotic cell death. This research revealed no significant difference in Bax mRNA expression among the groups. Furthermore, caspase 3 mRNA expressions were measured and showed no significant differences among the groups. These findings don’t match those observed from earlier studies suggesting that chlorogenic acid can reduce the expression of caspase 3\textsuperscript{19-21} However, these findings were observed in liver tissue\textsuperscript{19} and neuronal cell cultures\textsuperscript{20}.

The results of this study regarding the expression of apoptosis regulatory proteins Bcl2, Bax and caspase 3 are in accordance with our previous studies in the hippocampus area. In this study, it was found that chlorogenic acid increased the expression of Bcl2 mRNA, but did not affect the expression of Bax and caspase 3 in the rat hippocampus that experienced transient global ischemia.\textsuperscript{11}

Two pathways in apoptosis, i.e., intrinsic and extrinsic pathways, involve caspases. However, there is also a caspase-independent pathway of apoptosis in which caspases are not involved. Caspase-independent apoptosis is initiated by the release of apoptosis-inducing factor (AIF) and endonuclease G (endoG) to the nucleus of the cell from the mitochondria, then triggers DNA damage.\textsuperscript{25} During brain ischemia, AIF will be released in response to poly ADP-ribose polymerase 1 (PARP1) activation and induce cell death.\textsuperscript{26-28} AIF was released earlier than cytochrome C.\textsuperscript{27} This might explain why there was no difference in caspase 3 mRNA expression between groups.

Another possible explanation is the necrotic pathway of cell death. In conditions of cerebral ischemia, free radicals are formed, which causes an increase in Ca\textsuperscript{2+} influx. When neurons experience energy deficiency, such as during ischemia, ion homeostasis is disrupted.\textsuperscript{29} Cell death via the necrotic pathway is highly likely to occur under severe cellular energy depletion conditions. This is attributed to the fact that the apoptosis pathway requires energy to proceed.\textsuperscript{25} This study induced a global ischemia for 20 minutes, resulting in a significant reduction in energy supply. The necrotic pathway does not involve the activation of the apoptosis cascade, thereby not affecting apoptosis markers.\textsuperscript{30} Previous research stated that the influx of Ca\textsuperscript{2+} into cells subsequently triggers the activation of apoptosis.\textsuperscript{30} CGA suppresses the calcium entry into cells through N-methyl D-aspartate (NMDA) receptors.\textsuperscript{31} Inhibition of calcium entry prevents activation of proapoptotic proteins, hence protecting cells from death. This explains why, in the present study, CGA did not impact Bax and caspase. CGA prevents cell death by inhibiting calcium entry and increasing Bcl2.

This study showed that the dose of chlorogenic acid that increased Bcl2 mRNA expression and NeuN expression was the highest dose, namely 60 mg/kgBW. At lower doses, namely 15 mg/kg and 30 mg/kg, there was no significant difference with the ischemia group without chlorogenic acid. The protective effect of chlorogenic acid against neuronal cell death in the cerebellum after ischemia reperfusion injury turned out to be dose dependent. This research can be continued with pre-clinical tests in the form of toxicity tests, and then clinical trials in humans can be carried out. This research focused on observing neuroprotective abilities of CGA at the molecular level by utilizing the NeuN and the apoptosis process gene markers. Considering that cell death does not occur solely through the apoptotic pathway, in this study, global ischemia induced for 20 minutes, significantly reducing energy supply. Therefore, it is strongly recommended that further studies be carried out using markers that reflect the necrotic processes (such as AMPA receptors activation), where the necrotic pathway tends to occur under conditions of very limited energy supply. Histological examination via stereological cell counting can also be performed to assess the direct effects on cells.

Conclusion

Chlorogenic acid in 60 mg/kgBW dose increases NeuN expression and Bcl2 mRNA expression after transient global ischemia. These increases might correlate with the heightened level of protection against apoptosis in cerebellum, hence showing its potential in protecting neuron cells in the cerebellum.

Acknowledgements

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Authors Contribution

Concepting, planning, data collection, review manuscript and approved the final version of the manuscript were performed by all authors. E.H. and M.H. analyzed the data. E.H. drafted the original manuscript.

References