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Spirulina Prevent Caspase-Independent Apoptosis in the Cochlea and Brainstem of Senescence-Accelerated Prone-8 Mice

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Abstract

Background: Spirulina platensis water extract (SP) could decrease hearing degeneration via reducing oxidative stress damages in the auditory system of senescence-accelerated prone-8 (SAMP8) mice. This study aimed to investigate the effects of SP on the caspase-independent apoptosis in the cochlea and brainstem of SAMP8 mice.

Methods: Twelve 11-month-old SAMP8 mice were randomly divided into two groups: control group (SAMP8 mice was fed a normal diet) and spirulina group (SAMP8 mice was fed a normal diet with oral supplementation SP for 6 weeks. Auditory brainstem responses (ABRs) were measured in the beginning and at the end of the study. Cochlear histology and immunochemistry and Western blotting of brainstem were performed at the end of the study.

Results: Compared with control group, spirulina group had significantly lower ABR thresholds using click sound stimulation at the end of this study. The spirulina group had significantly higher counts of outer hair cells (OHC) and spiral ganglion neuron (SGN) density in the middle turn of the cochlea. The spirulina group had significantly lower expressions of poly (ADP-ribose) polymerase-1 (PARP-1) and apoptosis-inducing factor (AIF) in the cochlea. Also, the spirulina group had significantly lower expression of PARP-1, but not the AIF, in the brainstem.

Conclusions: SP could decrease hearing degeneration in SAMP8 mice possibly via reducing caspase-independent apoptosis signal pathway in the cochlea and brainstem.

Background

Age-related hearing impairment (ARHI) was common in older subjects. Both of peripheral and central auditory pathways degenerated during aging [1]. ARHI was a complex disease and many factors would contribute to its severity. For example, genetic background, noise exposure, obstructive sleep apnea, metabolic syndrome, ototoxic agents, etc. [2–14]. Hypoxia, neural inflammation, oxidative stress damages, and caspase-dependent cell apoptosis in the auditory system were the most known mechanisms for ARHI [8, 15–18]. However, caspase-independent cell apoptosis in ARHI was rarely reported till now.

Caspase-dependent apoptosis signal pathway was the well-known mechanism of cell death in the auditory system. For example, Riva et al. (2007) reported that increased hypoxia, oxidative stress, inflammation, and caspase-dependent apoptosis signal pathway was found in the cochlea of CD/1 mice. On the contrary, caspase-independent apoptosis signaling pathway was relatively less discussed. Poly (ADP-ribose) polymerase-1 (PARP-1), apoptosis inducing factor (AIF), and endonuclease G (EndoG) play key roles in the caspase-independent apoptosis signaling pathway. If DNA was severely damaged, activated PARP-1 would enter mitochondria and activated AIF releasing into the cytosol and nucleus. Thereafter, EndoG was activated and finally resulted in cell death [19]. Such caspase-independent apoptosis signaling pathway played a key role in several animal and human diseases, including

neurodegenerative diseases [20–22], and ischemia/reperfusion-related brain damages [19, 23]. Our previous study also showed that diet-induced obesity increased cell loss in the cochlea via activation of both caspase-dependent and -independent apoptosis signaling pathways in mice [8].

Spirulina platensis, one type of blue-green algae, had anti-inflammatory and antioxidative effects via inhibiting cyclooxygenase-2 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes [24, 25]. Our previous study had showed that spirulina platensis water extract (SP) could decrease hearing degeneration via modulating expressions of endogenous antioxidant genes and reducing oxidative stress damages in the cochlea and brainstem of senescence-accelerated prone-8 (SAMP8) mice [26]. However, the effects of SP on the caspase-independent apoptosis in the auditory system was still unknown. Therefore, we aimed to investigate this issue in this study in SAMP8 mice.

Material And Methods

Animals

Twelve 11-month-old male SAMP8 mice were randomly divided into two groups: the control group was fed a normal diet (Fwusow Industry Co, Ltd, Taiwan), and the spirulina group was fed a normal diet with oral supplementation SP (400 mg/kg body weight) for 6 weeks. The animals were housed (in groups of four mice per cage) in a temperature-controlled room with a constant 12-hr light–dark cycle. Food and tap water were freely available throughout the experiments. The Institutional Animal Care and Use Committee of Dalin Tzu Chi Hospital approved the protocol used in this study.

Preparation of SP

SP from Far East Bio-tec Co, Ltd (Taipei, Taiwan) was prepared as follows: suspension of S. platensis powder and pure water was disrupted for 24 hours and centrifuged. The supernatant was collected and lyophilized. The lyophilized SP contained 35–45% polysaccharides, 15–25% phycobiliproteins (C-phycocyanin and allophycocyanin), 10–20% proteins other than phycobiliproteins, 10–12% ash and 5–8% water. Among that, the active compounds in the extract were sulfated polysaccharides and phycobiliproteins.

Auditory brainstem responses (ABRs)

ABRs (Intelligent Hearing Systems, Miami, FL) were measured at the start (11 months of age) and at the end of the study under general anesthesia with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Click sounds, 8- and 16-kHz tone bursts were delivered sequentially to the left ear through earphones (Telephonics Corp, Farmingdale, NY). Subdermal needles were used for recording. The active electrode was inserted at the vertex; the reference electrode was ventrolateral to the left ear; and the

ground electrode to the low back above the tail. The amplified responses were then averaged by a computer and displayed on a computer screen.

ABR thresholds were obtained by reducing the stimulus intensity in 5-dB intervals and increasing the stimulus intensity in 3-dB intervals to identify the lowest intensity at which ABR waves I-V were detected by one well-trained audiologist who was blinded to all groups. The ABR data were stored digitally on disks for offline measurements and analysis of latency of ABR components later.

Histology and immunochemistry of the cochlea

At the end of the study, the mice were sacrificed by decapitation under general anesthesia with an intraperitoneal injection of pentobarbital (65 mg/kg). Before being euthanized, animals were perfused of 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffered saline (PBS: NaCl, 150 mM; KH_2PO_4, 2 mM; Na_2HPO_4 $2H_2O$, 8 mM, pH 7.4) via an intracardiac injection. For each mouse, the temporal bones were removed, and the cochlea was perfused with the above fixative through the oval and round windows to an outlet within the apex. After 24-hr post-fixation in the same fixative at 4 °C, the temporal bones were decalcified in 10% EDTA solution, 4% paraformaldehyde, and 2.5% glutaraldehyde in PBS (pH 7.4) for 1 week at room temperature. The temporal bones were then rinsed in PBS, dehydrated through a graded series of alcohol and xylene, and embedded in paraffin.

For histologic staining

Embedded cochleae were sectioned at 5-μm thickness at the mid-modiolar level. The cochlear structures were observed with hematoxylin and eosin (H & E) staining using a light microscope. The cell density of neurons in SG, within a central region of 50 × 50 μm^2 at the middle turn of cochlea, were measured under × 400 magnification. The numbers of SGN were counted by the presence of stained nucleus. The morphology and numbers of OHCs and inner hair cells (IHCs) in the Organ of Corti of cochlea were observed under × 400 magnification.

For immunochemical staining

The sections were heat-treated at 95 °C in 10 mM sodium citrate buffer for 20 min, incubated in 3% H_2O_2 for 10 min at room temperature, and blocked in 5% skim milk to prevent non-specific labeling. The specimens were then incubated overnight at 4 °C with one of the following primary antibodies: rabbit monoclonal anti-PARP-1 (1:500 dilution) (Abcam plc, Cambridge, U.K.), and rabbit anti- AIF (1:1000 dilution) (R&D systems, Inc., U.S.A.), were obtained from Cell Signaling Technology (TAIGEN, TPE, R.O.C.).

Slides were washed in PBS three times, exposed to a secondary antibody for 1 h at room temperature, and stained using the DAB staining kit (R&D systems, Inc., U.S.A.) according to the manufacturer's

protocol. Slides were dehydrated, mounted, and observed through a light microscope. The images were acquired using a CCD camera (Dage-MTI Inc, Michigan City, Ind) connected to a personal computer and analyzed using image analysis software (Image-Pro Plus, version 6.0; Media Cybernetics, Silver Springs, MD).

The above features were observed blindly in two adjacent cochlear sections at 10-slides apart (equals to 50 μm apart) at the same mid-modiolar level. All data were acquired from two cochleae and averaged for each mouse by one well-trained researcher. Stained regions were quantified as the percentage of the entire target area.

Western blotting

The brainstem tissue of SAMP8 mice were dissected cut into small pieces (50–60 mg), homogenized with an appropriated volume of ice-cold lysis buffer with protease/phosphatase inhibitor cocktail, and incubated in the ice for 10 min. Cytoplasmic extraction reagent was added to the tube and centrifuged for 5 min (~ 16,000 xg). The supernatant was immediately transferred to a clean tube (cytoplasmic extract), and added the nuclear extraction reagent to the insoluble fraction. The tube was placed on ice and continued vortexing for 15 seconds every 10 minutes, for a total of 40 minutes, and centrifuged for 10 minutes (~ 16,000 xg). The supernatant was immediately transferred to another clean tube (nuclear extract). Total protein in the supernatant was measured by the BCA method (Bio-Rad, Hercules, CA). Equal amount of protein (20–50 μg) were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were washed with TBST buffer (10 mM Tris-HCl, 150 mM NaCl; pH 7.5) supplemented with 0.1% Tween 20 and then blocked with TBST containing 5% nonfat dried milk. The membranes were incubated overnight with primary antibodies. After being washed three times with TBST, the membranes were exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at room temperature. The membranes were washed three times with TBST at room temperature. Immunoreactivity was detected using ECL reagents. Anti-cleavaged PARP1 and anti-PARP1 (1:500, Boster, CA), anti-AIF (1:10000, Boster, CA), and anti-GADPH (1:50000, Boster, CA) antibodies, were obtained from Cell Signaling Technology (TAIGEN, TPE, ROC). Densitometric analysis of the data was performed using FusionCapt Advance Camera and FusionCapt Advance analyzer ver. 16.07 software.

Statistical analysis

ABR thresholds; counts of OHCs and IHCs, SGN cell density, ratios of PARP-1 and AIF staining in the cochlea, ratios of cleavage PARP-1versus PARP-1 protein expressions, and AIF versus GADPH protein expressions in the brainstem, were compared between two groups using a Student’s t test with Welch’s approximation. All analyses were performed using STATA 10.0 software (Stata Corp, LP, College Station, TX). P values of < 0.05 were considered significant.

Results

Table 1 showed the ABR thresholds at the beginning and at the end of this study. The ABR thresholds were not significantly different between two groups in click sound Stimulation (61.5 ± 4.9 versus 63.8 ± 3.5 dB SPL, p = 0.3100), 8-kHz tone burst stimulation (46.1 ± 4.7 versus 45.8 ± 5.1 dB SPL, p = 0.8813), or 16-kHz tone burst stimulation (64.1 ± 1.8 versus 65.8 ± 3.4 dB SPL, p = 0.2913) at the beginning of this study. However, the ABR thresholds were significantly lower in the spirulina group (64.8 ± 3.5 dB SPL) than in the control group (68.9 ± 4.2 dB SPL) (p = 0.0496) in click sound stimulation at the end of this study. Otherwise, the ABR thresholds were not significantly different between two groups in 8-kHz tone burst stimulation (52.8 ± 3.7 versus 49.6 ± 5.5 dB SPL, p = 0.2022) or 16-kHz tone burst stimulation (70.5 ± 3.7 versus 70.5 ± 3.8 dB SPL, p = 0.6956) at the end of this study.

Figure 1 showed the results of cochlear histology at the end of this study. Compared to the control group, the spirulina group had significantly higher counts of OHC (2.8 ± 0.2 versus 2.4 ± 0.4, p = 0.0143) in the middle turn of the cochlea. But, the cell counts of IHC were not significantly different in the control group (0.9 ± 0.2) and the spirulina group (1.0 ± 0.1) (p = 0.0898) (Fig. 1a). The SGN density was significantly higher in the spirulina group (18.9 ± 3.9 /50 × 50 μm^2) than in the control group (15.0 ± 4.7 / 50 × 50 μm^2) (p = 0.0476) in the middle turn of the cochlea (Fig. 1b).

Figure 2 showed the results of immunochemistry in the cochlea at the end of this study. Compared to the control group, the spirulina group had significantly lower expressions of PARP-1 in the IHC (0.30 ± 0.06 versus 0.72 ± 0.11, p < 0.0001), OHC (0.42 ± 0.11 versus 0.69 ± 0.14, p = 0.0008), and SGN (0.43 ± 0.05 versus 0.71 ± 0.09, p < 0.0001) (Fig. 2a).

Also, the spirulina group had significantly lower expressions of AIF in the IHC (0.38 ± 0.09 versus 0.64 ± 0.22, p = 0.0066), OHC (0.40 ± 0.17 versus 0.73 ± 0.09, p = 0.0002), and SGN (0.44 ± 0.19 versus 0.64 ± 0.14, p = 0.0280) (Fig. 2b).

Figure 3 showed the results of Western blotting in the brainstem at the end of this study. Compared to the control group, the spirulina group had significantly lower ratio of cleavaged PARP-1 versus PARP-1 expressions (0.78 ± 0.05 versus 0.99 ± 0.11, p = 0.0015) in the brainstem. But, the ratio of AIF versus GADPH expressions was not significantly different in the control group (1.10 ± 0.37) and the spirulina group (0.83 ± 0.17) (p = 0.1398).

Discussion

This animal study showed that diet supplementation of SP could prevent hearing degeneration in SAMP8 mice. SP could reduce losses of OHC and SGN in the cochleapossibly via reducing caspase-independent apoptosis signal pathway. Also, SP might reduce the caspase-independent apoptosis signal pathway in the brainstem.

Since oxidative stress damage was the important mechanism for ARHI, many exogenous antioxidants were used to prevent ARHI in animals and humans. For example, vitamins C and E, and caffeine might prevent hearing degeneration and/or auditory neuropathy in animals [16, 27, 28]. Folic acid could reduce hearing degeneration in subjects with lower folic acid intake [25]. Tea drinking was also associated with better hearing sensitivity in aged subjects [29]. In this study, we had chosen SAMP8 mice as an animal model for ARHI. Increased oxidative stress damages, decreased endogenous antioxidative capabilities, increased inflammation and apoptotic and/or autophagic cell death might account for premature hearing loss in SAMP8 mice [29]. On the contrary, SP could slow down the hearing degeneration in SAMP8 mice as shown in this study.

Previous studies determined that activation of the redox system occurred initially at the plasma membrane, in which NADPH oxidase could reduce oxygen to superoxide anion radicals. Thereafter, excess free radicals and/or reduced protection from antioxidants might be destructive to molecules in the subcellular and cellular levels, and subsequent resulted in cell death. So, it was very reasonable to see that SP, as an inhibitor for NADPH oxidase enzymes, could reduce oxidative stress damages and prevent diseases [24]. For example, SP could reduce oxidative damages in the brain and prevent memory loss in SAMP8 mice [31]. SP could decrease salicylate-induced tinnitus by reducing oxidative stress damages and neuroinflammation in many brain regions of mice [32, 33]. Our previous study also demonstrated that SP could prevent ARHI by increasing expressions of antioxidant genes and decreasing malondialdehyde levels in the cochlea and brainstem of SAMP8 mice [26].

Both of the caspase-dependent and -independent apoptosis signal pathways were involved in many animal and human diseases. For example, ischemic brain damages [23], neurodegenerative diseases [20], Parkinson's disease [21] were associated with neural cell deaths via these two apoptosis signal pathways. Our previous study also showed that diet-induced obesity increased cell loss in the cochlea via activation of both caspase-dependent and -independent apoptosis signaling pathways in mice [8]. Now, we showed that caspase-independent apoptosis signaling pathways might contribute to ARHI in SAMP8 mice and SP supplementation might prevent ARHI via reducing PARP-1 expression in the cochlea.

Conclusions

The beneficial effects of SP on auditory functions might be associated with preventing OHC and SGN cell losses and possibly via decreasing caspase-independent apoptosis signal pathway in the cochlea and brainstem.

Declarations

Ethics approval and consent to participate:

The Institutional Animal Care and Use Committee of Dalin Tzu Chi Hospital approved the protocol used in this study.

Consent for publication:

Not applicable

Availability of data and materials:

Not applicable

Competing interests

The authors declare that they have no competing interests

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Author Contributions:

Conceptualization, J.H.H. and Y.C.C.; Methodology, J.H.H.; Software, Y.C.C.; Validation, M.H.L.; Formal Analysis, M.H.L.; Investigation, M.H.L.; Resources, Y.C.C.; Data Curation, M.H.L.; Writing – Original Draft Preparation, J.H.H. Writing – Review & Editing, J.H.H.

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Table

Table 1. ABR thresholds at the beginning and the end of this study.

Mean±SD (dBSPL)	Control group	Spirulina group	p values
At the beginning			
Click sound	61.5±4.9	63.8±3.5	0.3100
8 kHz tone burst	46.1±4.7	45.8±5.1	0.8813
16 kHz tone burst	64.1±1.8	65.6±3.4	0.2913
At the end			
Click sound	68.9±4.2	64.8±3.5	0.0496
8 kHz tone burst	52.8±3.7	49.6±5.5	0.2022
16 kHz tone burst	71.3±3.7	70.5±3.8	0.6956

Abbreviations: SD: standard deviation; ABR: auditory brainstem response. dBSPL: decibel sound pressure level.

Figures

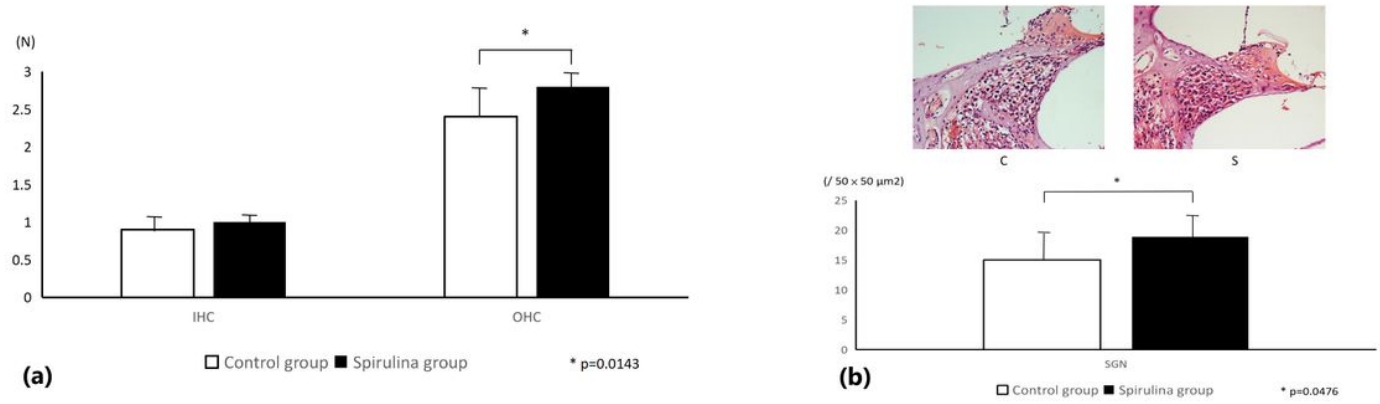

Figure 1

Results of cochlear histology at the end of this study. Compared to the control group, the spirulina group had significantly higher counts of OHC (p= 0.0143) in the middle turn of the cochlea. But, the cell counts of IHC were not significantly different in both group (p= 0.0898) (Figure 1a). The SGN density was significantly higher in the spirulina group than in the control group in the middle turn of the cochlea (p=0.0476) (Figure 1b).

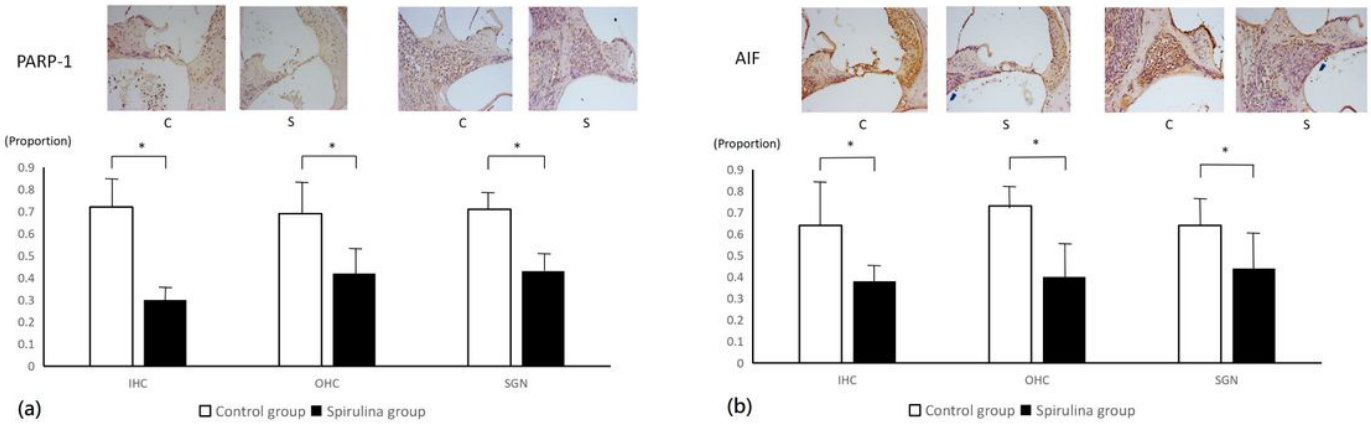

Figure 2

Results of immunochemistry in the cochlea at the end of this study. Compared to the control group, the spirulina group had significantly lower expressions of PARP-1 in the IHC (p <0.0001), OHC (p= 0.0008), and SGN (p<0.0001) (Figure 2a). Also, the spirulina group had significantly lower expressions of AIF in the IHC (p=0.0066), OHC (p= 0.0002), and SGN (p=0.0280) (Figure 2b).

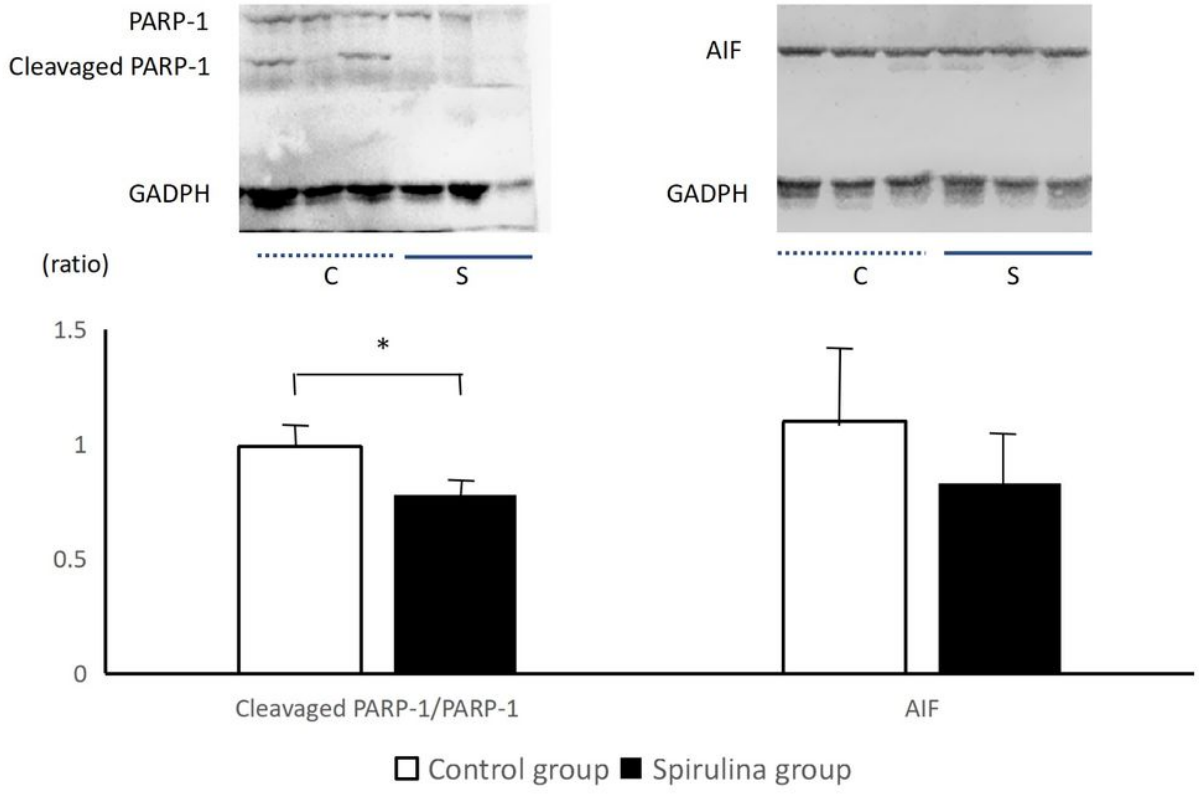

Figure 3

Results of Western blotting in the brainstem at the end of this study. Compared to the control group, the spirulina group had significantly lower ratio of cleavaged PARP-1 versus PARP-1 expressions (p= 0.0015). But, the ratio of AIF versus GADPH expressions was not significantly different in both groups (p=0.1398).