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Original Article

Fucoidan Extract from *Laminaria religiosa* Suppresses Ischemia-induced Apoptosis and Cell Proliferation in the Hippocampus of Gerbils

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Fucoidan has been shown to exhibit a host of biological activities, including anti-coagulant, anti-thrombotic, anti-tumourigenic, anti-inflammatory, anti-viral, anti-complementary and neuroprotective effects. In the present study, we attempted to determine the effects of *Fucoidan* on both apoptosis and cell proliferation in the hippocampal CA1 region and the dentate gyrus of gerbils after the induction of transient global ischemia. This experiment involved the use of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as well as immunohistochemistry for caspase-3 and 5-bromo-2'-deoxyuridine (BrdU). The monosaccharide composition of the purified *Fucoidan* which had been extracted from *Laminaria religiosa* was utilized in this study. The present study clearly induces that apoptotic cell death and cell proliferation in the gerbil's hippocampal regions increased significantly following the induction of transient global ischemia and the results of this study also indicate that *Fucoidan* exerted a suppressive effect on this observed ischemia-induced increase in apoptosis within the CA1 and dentate gyrus, and also suppressed cell proliferation in the dentate gyrus.

Key Words : *Fucoidan*, transient global ischemia, apoptotic cell death, cell proliferation, *Laminaria religiosa*, neuroprotective effect

Introduction

Fucoidan, a sulfated polysaccharide which can be extracted from brown algae, is possessed of a fairly complex structure. Fucoidan polysaccharides contain large proportions of L-fucose and sulfate, with minor amounts of other sugars, including xylose, galactose, mannose, and glucuronic acid[1]. *Fucoidan* has been shown to exert a variety of biological effects, including anti-coagulant, anti-thrombotic, anti-tumourigenic, anti-viral, anti-complementary, anti-inflammatory activities, and neuroprotective effects[2,3].

Cerebral ischemia is a result of a reduction in cerebral blood flow, usually due to the transient or permanent occlusion of cerebral arteries[4]. Ischemic injuries in the brain lead to neuronal cell death, and eventually culminate in neurological impairments[5,6]. Pyramidal neurons in the hippocampal CA1 region are known to be particularly vulnerable to ischemic injury[7].

Apoptosis, which is also commonly referred to as programmed cell death, is a form of cell death which functions in the elimination of dying cells within proliferating or differentiating cell populations[8]. Thus, apoptosis plays a vital

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role in both normal development and tissue homeostasis[9]. However, inappropriate or excessive apoptosis has also been implicated in the development of several neurodegenerative disorders, including ischemia[10]. Apoptotic cell death is commonly assessed via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, which detects the fragmentation of DNA. Another important characteristic of apoptosis is caspase-3 activation. Caspase-3 is the most extensively studied member of the caspase family, and is one of the key executors of apoptosis[11]. In ischemic animal models, caspase-3 activation has been implicated in apoptotic death of neuronal cells[5].

Neurogenesis occurring in the hippocampus performs a vital function in the processes inherent to learning and memory. Neurogenesis continues throughout life in the brain of adult mammals, including humans[12]. Cell proliferation in the hippocampal dentate gyrus has been shown to increase as the result of learning, serotonin, N-methyl-D-aspartate (NMDA) receptor antagonists, and exposure to enriched environments[13]. Increased cell proliferation has also been observed in the hippocampal dentate gyrus in several pathological states, including seizure, mechanical lesions of the dentate gyrus lesions, and ischemic injuries. Such up-regulations in cell proliferation during ischemic insult are considered to present a compensatory adaptive response to increased apoptotic neuronal cell death after the infliction of an ischemic injury [14].

In the present study, the effects of *Fucoidan* on cell proliferation and apoptosis in the hippocampal regions after the induction of transient global ischemia in gerbils were determined via TUNEL assay and immunohistochemistry for caspase-3 and 5-bromo-2'-deoxyuridine (BrdU).

Materials and Methods

1. Drugs and reagents

The *Fucoidan* utilized in this study was extracted from *Laminaria religiosa*, collected at the Wando area in Korea. Commercial *Fucoidan*, L-fucose, D-galactose, D-xylose and D-mannose were purchased from the Sigma Chemical Co. (St. Louis, MO), and all of the other chemicals, reagents, and solvents were of ACS grade.

2. HPAEC-PAD analysis

Dried sea tangle samples (100 g) were chopped into pieces of the appropriate size, suspended in 1 L of 0.01 N HCl, and then allowed to stand for 24 h at room temperature. The acid extract was neutralized with 10 N NaOH, precipitated with 3 volumes of absolute ethanol, and then maintained for 24 h at 4°C. After 20 min of centrifugation at 7,000 X g at 4°C, the pellets were dissolved in deionized distilled water (ddH2O), and were precipitated at a pH of 2, with 2 M CaCl2. The precipitates were then resuspended in ddH2O and were precipitated with 3 volumes of absolute ethanol with gentle agitation. The pellets were resuspended in ddH2O and dialyzed (MWCO 6,000, Spectrapor Dialysis Membrane, Spectrum Co., Gardena, CA) against ddH2O at 4°C for 2 days. The dialysates were collected and lyophilized. The carbazole method as described by Bitter and Muir[15] was then conducted, and measurements taken with a spectrophotometer (SECOMAM, Ales, France). Ten mg of dried *Fucoidan* samples were then acid-hydrolyzed with 2 M trifluoroacetic acid (TFA), in screw- capped microfuge tubes. After 4 h of acid hydrolysis at 100 °C, the solutions were dried with a Speed-Vac (Module spin 40, Biotron, Korea) in order to remove the acid, and were dissolved in 400 μ l of

ddH2O. The hydrolysates were then applied to high-performance anion-exchange chromatography, coupled with a pulsed amperometric detection system (HPAEC-PAD) for the monosaccharide compositions. The HPAEC-PAD system consisted of a Bio-LC (Dionex, Sunnyvale, CA), which has been equipped with a pulsed amperometric detector (PAD-II), and a gold working electrode. The neutral sugars were isocratically eluted for 40 min from a CarboPac PA-1 column (4 × 250 mm) at a flow rate of 1.0 ml/min, with a mixture of 9% 200 mM NaOH and 91% water. The data were then collected and analyzed using Chromeleon TM software (Dionex, UK).

3. Experimental animals

Adult male Mongolian gerbils (11 - 13 weeks of age) were used as the model animal for this experiment. All of the experimental procedures in this study were performed in accordance with the animal care guidelines established by the National Institutes of Health (NIH) as well as the Korean Academy of Medical Sciences. The gerbils were housed under controlled temperature (20 ± 2 °C) and lighting (07:00 to 19:00 h) conditions with food and water made available *ad libitum*. The gerbils were then divided randomly into five groups ($n = 6$ in each group): the sham-operation group, the ischemia-induction group, the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and the ischemia-induction and 200 mg/kg *Fucoidan*-treated group.

Beginning on the second day after the operation, the *Fucoidan* was administrated at the indicated dosage to the gerbils by oral lavage, once a day for 7 consecutive days. The sham-operation group and the ischemia induction group received water once a day orally, for the same duration.

BrdU (50 mg/kg; Sigma Chemical Co., St. Louis, MO) was intraperitoneally administrated to all of the animals, 1 h before the beginning of Fucoidan treatment, once a day for 7 days. BrdU, a thymidine analogue, is incorporated into the DNA of the dividing cells and can be detected via immunohistochemistry[16].

4. Surgical procedure

In order to induce transient global ischemia in the gerbils, a surgical procedure predicated on the previously described experimental method [14, 17] was conducted. In brief, the gerbils were anesthetized with 3% isoflurane in 20% O2-77% N2. After making bilateral neck incisions, both of the common carotid arteries of the gerbils were exposed, and were then occluded via the application of aneurysm clips for 7 min. The clips were removed, restoring cerebral blood flow. The gerbils' rectal temperatures were maintained at 37 ± 0.5°C with a heating lamp, until the gerbils had regained consciousness. After recovery, each of the animals was monitored for an additional 2 h, in order to prevent hypothermia. The animals in the sham- operation group were treated in an identical fashion, except that the common carotid arteries were not occluded during the neck incisions.

All gerbils were sacrificed on the 7th day of the experiment. The animals were anesthetized with Zoletil 50 □ (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains of the gerbils were then dissected and postfixed overnight in the same fixative, and transferred into a 30% sucrose solution for cryoprotection.

Coronal sections of 40 μ m thickness were made with a freezing microtome (Leica, Nussloch, Germany).

5. TUNEL staining

In order to visualize DNA fragmentation, a known marker for apoptotic cell death, TUNEL staining was conducted using an In Situ Cell Death Detection Kit □ (Roche, Mannheim, Germany), in accordance with the manufacturer's protocols[17]. At the beginning of the procedure, the sections were post-fixed in ethanol-acetic acid (2:1) and then rinsed. The sections were then incubated with proteinase K (100 μ g/ml), rinsed, incubated with 3% H2O2, permeabilized with 0.5% Triton X-100, rinsed again, and then incubated in a TUNEL reaction mixture. The sections were rinsed and visualized with Converter-POD, supplemented with 0.02% 3,3'- diaminobenzidine (DAB). Mayer's hematoxylin (DAKO, Glostrup, Denmark) was utilized for counterstaining. Finally, the sections were mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and the coverslips were mounted using Permount □ .

6. Caspase-3 immunohistochemistry

In order to visualize the expression of caspase-3 expression, caspase-3 immunohistochemistry was conducted as previously described[17]. The sections were prepared from each of the brains, and were incubated overnight with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and then for another 1 h with biotinylated mouse secondary antibody (1:100 Vector Laboratories, Burlingame, CA, USA). Bound secondary antibody was then amplified using the Vector Elite ABC kit □ (Vector Laboratories). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% DAB and the sections were finally mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and the coverslips were mounted using Permount □ .

7. BrdU immunohistochemistry

In order to detect the newly generated cells in the dentate gyrus, BrdU-specific immunohistochemistry was conducted as previously described[17]. The sections were initially permeabilized via incubation in 0.5% Triton X-100 in PBS for 20 min, and were then pretreated for 2 h with 50% formamide-2 x standard saline citrate (SSC) at 65°C, denaturated for 30 min in 2 N HCl at 37°C, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4°C with BrdU-specific mouse monoclonal antibody (1:600; Roche, Mannheim, Germany). The sections were then washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories). The sections were then incubated for another 1 h with avidin-peroxidase complex (1:100; Vector Laboratories). For visualization, the sections were incubated in 50 mM Tris-HCl (pH 7.6) containing 0.02% DAB, 40 mg/ml nickel chloride, and 0.03% hydrogen peroxide for 5 min, and the sections were finally mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount □ .

8. Image analysis

The numbers of TUNEL-positive cells and caspase-3-positive cells in the CA1 and dentate gyrus, and the number of BrdU-positive cells in the dentate gyrus, were counted using an Image-Pro □ Plus computer-assisted image analysis

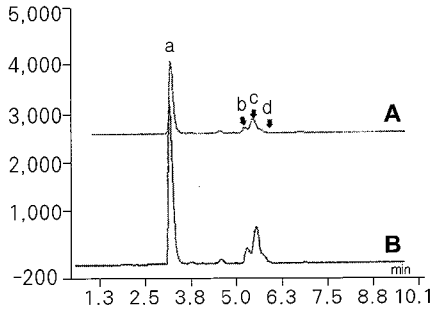

Fig. 1. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) analysis for the degree of purity of the *Fucoidan*
Acid hydrolysis of the purified *Fucoidan* was performed with 2 M trifluoroacetic acid for 4 h at 100°C and the hydrolysates were analysed by HPAEC-PAD using Bio-LC as described in the text.
(a) L-fucose (b) D-mannose (c) D-xylose (d) D-galactose
(A) hydrolysate of *Fucoidan* (used in this study) (B) hydrolysate of commercial *Fucoidan* as a reference

system (Media Cybernetics Inc., Silver Spring, MD, USA) attached to a light microscope (Olympus, Tokyo, Japan). The number of cells counted was expressed as the number of cells per square millimeter (mm2) of cross-sectional area in each of the selected hippocampal regions.

9. Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Duncan's post-hoc test, and the results were expressed as the mean ± standard error mean (SEM). Statistical significance considered at $P < 0.05$.

Results

1. Purity of the Fucoidan assay

The *Fucoidan* from *Laminaria religiosa* was purified primarily via ethanol and CaCl2 precipitation, and this resulted in a mass yield of 2.3%. The purity of the purified *Fucoidan* was evaluated via comparison of its monosaccharide composition to that of commercial standard *Fucoidan* (Sigma Chemical Co., St. Louis, MO, USA) after 2 M TFA hydrolysis, as is shown in Fig. 1. This experimental monosaccharide composition analysis indicated that the *Fucoidan* obtained was nearly completely identical to commercial standard *Fucoidan*, thereby suggesting that it had been purified to apparent homogeneity. Monosaccharide composition analysis also indicated this experimental *Fucoidan* was composed of fucose, xylose, galactose and mannose at molar ratios of 63.71, 22.98, 6.62, and 0.24, respectively, and also contained minor proportions of uronic acid (Fig. 1).

2. Effect of Fucoidan on DNA fragmentation in the hippocampal regions after transient global ischemia

In the hippocampal CA1 region, the number of TUNEL-positive cells was as follows: 6.04 ± 1.59/mm2 in the sham-operation group, 659.01 ± 159.78/mm2 in the ischemia-induction group, 461.63 ± 58.41/mm2 in the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, 367.04 ± 62.16/mm2 in the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and 261.29 ± 31.34/mm2 in the ischemia-induction and 200

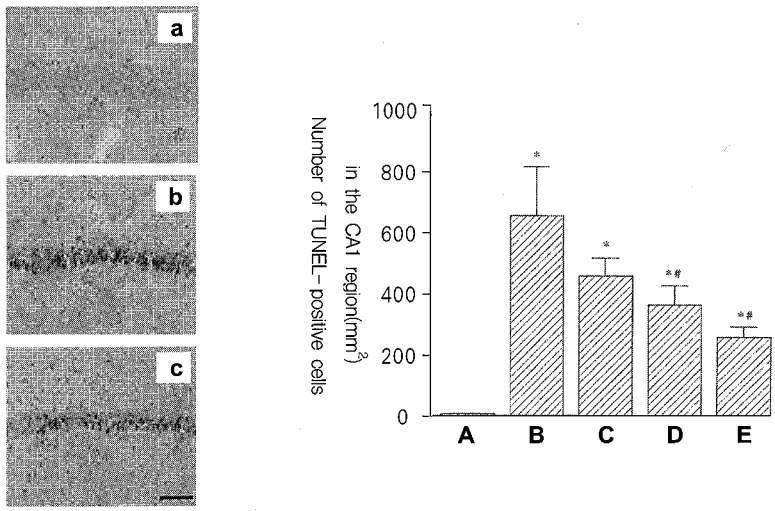

Fig. 2. Effect of *Fucoidan* on DNA fragmentation in the hippocampal regions after transient global ischemia
Upper : A photomicrograph of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells in the CA1 region
· The scale bar represents 100 ㎛.
(a) sham-operation group (b) ischemia-induction group
(c) ischemia-induction and 200 mg/kg *Fucoidan*-treated group
Middle : Mean number of TUNEL-positive cells in the CA1 region
Lower : Mean number of TUNEL-positive cells in the dentate gyrus
Values are represented as the mean ± SEM.
* represents $P < 0.05$ compared to the sham-operation group
# represents $P < 0.05$ compared to the ischemia-induction group
(A) sham-operation group (B) ischemia-induction group
(C) ischemia-induction and 50 mg/kg *Fucoidan*-treated group
(D) ischemia-induction and 100 mg/kg *Fucoidan*-treated group
(E) ischemia-induction and 200 mg/kg *Fucoidan*-treated group

mg/kg *Fucoidan*-treated group.

In the dentate gyrus, the number of TUNEL-positive cells was as follows: 3.87 ± 1.04/mm2 in the sham-operation group, 23.58 ± 2.57/mm2 in the ischemia-induction group, 14.50 ± 1.08/mm2 in the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, 12.35 ± 1.22/mm2 in the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and 9.91 ± 1.83/mm2 in the ischemia-induction and 200 mg/kg *Fucoidan*-treated group.

The results of this study clearly indicate that ischemic injuries resulted in an increase in the rate of apoptotic neuronal cell death occurring in the CA1 region and the dentate gyrus, and that *Fucoidan* exerted a significant suppressive effect on the ischemia-induced increase in apoptotic neuronal cell death occurring in the hippocampus (Fig. 2).

3. Effect of *Fucoidan* on caspase-3 expression in the hippocampal regions after transient global ischemia

In the hippocampal CA1 region, the number of caspase-3-positive cells was as follows: 10.19 ± 3.59/mm2 in the sham-operation group,

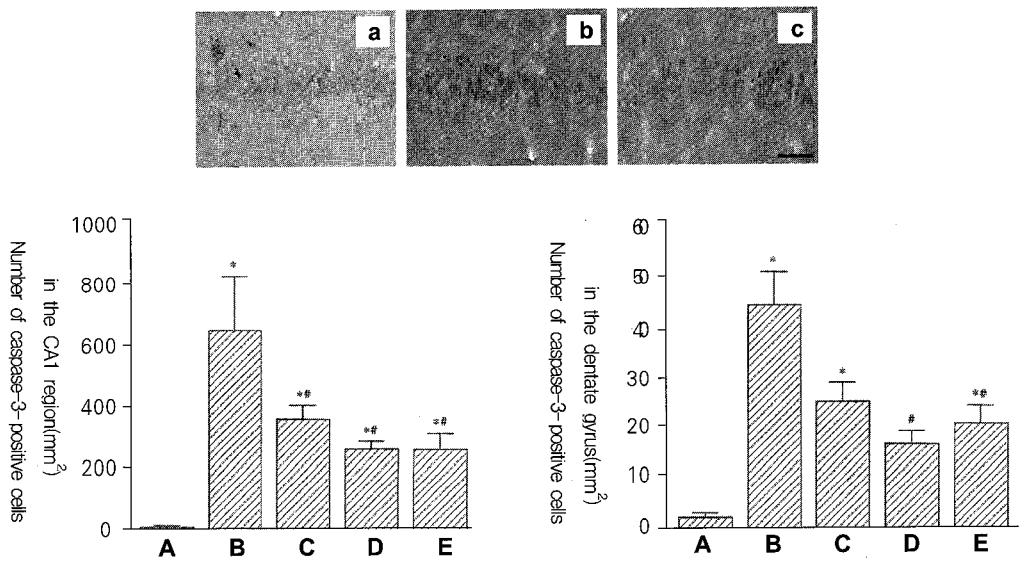

Fig. 3. Effect of *Fucoidan* on caspase-3 expression in the hippocampal regions after transient global ischemia
Upper : A photomicrograph of caspase-3-positive cells in the CA1 region
The scale bar represents 100 ㎛.
(a) sham-operation group (b) ischemia-induction group
(c) ischemia-induction and 200 mg/kg *Fucoidan*-treated group.
Middle : Mean number of caspase-3-positive cells in the CA1
Lower : Mean number of caspase-3-positive cells in the dentate gyrus
Values are represented as the mean ± SEM.
* represents $P < 0.05$ compared to the sham-operation group
# represents $P < 0.05$ compared to the ischemia-induction group
(A) sham-operation group (B) ischemia-induction group
(C) ischemia-induction and 50 mg/kg *Fucoidan*-treated group
(D) ischemia-induction and 100 mg/kg *Fucoidan*-treated group
(E) ischemia-induction and 200 mg/kg *Fucoidan*-treated group

664.04 ± 180.62/mm2 in the ischemia-induction group, 368.68 ± 46.55/mm2 in the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, 264.60 ± 25.57/mm2 in the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and 266.67 ± 52.66/mm2 in the ischemia-induction and 200 mg/kg *Fucoidan*-treated group.

In the dentate gyrus, the number of caspase-3-positive cells was as follows: 2.09 ± 0.90/mm2 in the sham-operation group, 44.67 ± 6.81/mm2 in the ischemia-induction group, 25.32 ± 3.84/mm2 in the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, 16.93 ± 2.57/mm2 in the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and 20.94 ± 3.54/mm2 in the ischemia-induction and 200 mg/kg *Fucoidan*-treated group.

The results of this study clearly indicate that ischemic injury enhances the expression of caspase-3 expression occurring in the CA1 region and the dentate gyrus, and that *Fucoidan* exerted a significant suppressive effect on the ischemia-induced increase in caspase-3 expression occurring

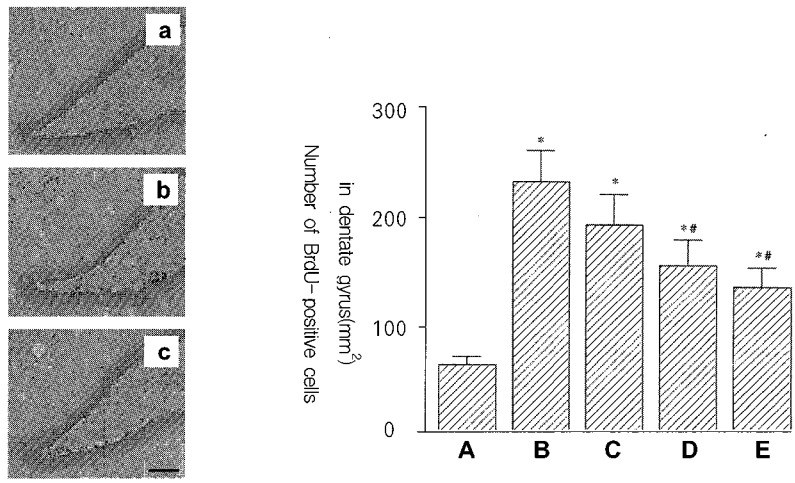

Fig. 4. Effect of *Fucoidan* on cell proliferation in the dentate gyrus after transient global ischemia
Upper : Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the dentate gyrus Sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown)
The scale bar represents 100 ㎛.
(a) sham-operation group (b) ischemia-induction group
(c) ischemia-induction and 200 mg/kg *Fucoidan*-treated group
Lower : Mean number of BrdU-positive cells in the dentate gyrus
Values are represented as the mean ± SEM.
* represents P < 0.05 compared to the sham-operation group
# represents P < 0.05 compared to the ischemia-induction group
(A) sham-operation group (B) ischemia-induction group
(C) ischemia-induction and 50 mg/kg *Fucoidan*-treated group
(D) ischemia-induction and 100 mg/kg *Fucoidan*-treated group
(E) ischemia-induction and 200 mg/kg *Fucoidan*-treated group

in the hippocampus (Fig. 3).

4. Effect of *Fucoidan* on cell proliferation in the dentate gyrus after transient global ischemia

The number of BrdU-positive cells in the dentate gyrus was as follows: 62.82 ± 6.99/mm2 in the sham-operation group, 235.69 ± 28.27/mm2 in the ischemia-induction group, 195.11 ± 27.89/mm2 in the ischemia-induction and 50 mg/kg *Fucoidan*-treated group, 157.63 ± 23.97/mm2 in the ischemia-induction and 100 mg/kg *Fucoidan*-treated group, and 137.47 ± 18.27/mm2 in the ischemia-induction and 200mg/kg *Fucoidan*-treated group.

The results of this study clearly indicate that ischemic injuries resulted in enhanced cell proliferation in the dentate gyrus, and that *Fucoidan* exerted a significant suppressive effect on the ischemia-induced increase in cell proliferation within the dentate gyrus (Fig. 4).

Discussion

Cerebral ischemia is one of the principal causes of death and disability in many countries, and can be induced by permanent or prolonged occlusion of arteries within the brain[18]. Cerebral

ischemia deprives oxygen and glucose in the brain tissue, which results in tissue infarction and neuronal cell death[5,6]. Pyramidal neurons in the hippocampal CA1 region have been shown to be particularly vulnerable to injuries induced by transient global ischemia, and neuronal cell death is frequently observed after the induction of ischemia[19].

Apoptosis has recently been suggested to constitute the chief form of cell death after ischemia. Apoptosis has been confidently implicated in several primarily ischemic and neurodegenerative diseases, including both Parkinson's disease and Alzheimer's disease[20]. The morphological characteristics of apoptosis are known to include cell shrinkage, chromatin condensation, membrane blebbing, internucleosomal DNA fragmentation, and the formation of apoptotic bodies, and all of these characteristics have been reported in cells currently undergoing ischemic death[6]. Caspase-3 up-regulation and activation of at the early stage apoptosis after ischemia has also been reported[5,17].

Fucoidan is known to inhibit leukocyte adhesion to L-selectin and P-selectin. P-selectin plays an important role in the pathogenesis of ischemic brain injury[21,22] and apoptosis[23]. Ruehl et al.[21] reported that *Fucoidan* reduces infarction size and improves brain neurological function. *Fucoidan* is known to increases blood flow[24], to enhance renoprotective activity[25], to facilitate angiogenesis[26], and to reduce infarct size following adult middle cerebral artery occlusion[21]. Uhm et al[3]. reported that *Fucoidan* inhibits ischemic neural cell death, and inhibiting activity on selectin-mediated leukocyte accumulation is an effective therapeutic intervention following ischemia insult. Klintman et al[23]. demonstrated that inhibition on P-selectin function reduces apoptosis.

In the present study, the numbers of TUNEL-positive cells and caspase-3-positive cells in the CA1 region and the dentate gyrus were significantly increased following ischemic attack, indicating that ischemia induces apoptotic neuronal cell death in the hippocampus. *Fucoidan* suppressed the ischemia-induced apoptotic neuronal cell death in the hippocampus.

Increased cell proliferation in the hippocampal dentate gyrus and cerebral cortex after ischemia has been previously documented[14,27]. Liu et al. [14] reported that cell proliferation in the dentate gyrus of gerbils is significantly increased after ischemia, and that it reaches the maximal level on the 11th day after ischemic episode. It is generally believed that ischemia-induced increment in cell proliferation is a compensatory response to excessive apoptotic cell death[14,27]. In the present study, markedly increased cell proliferation in the dentate gyrus was also observed following ischemic attack. *Fucoidan* suppressed the ischemia-induced cell proliferation in the dentate gyrus, in this study. Suppression of cell proliferation in the dentate gyrus can be ascribed to the reduction of apoptotic cell death by *Fucoidan*.

In this study, we have shown that apoptotic cell death in the hippocampus and cell proliferation in the dentate gyrus were significantly increased following transient global ischemia in gerbils and that *Fucoidan* treatment suppressed the ischemia-induced increase in apoptosis in the hippocampus and cell proliferation in the dentate gyrus. The present results suggest that *Fucoidan* overcomes the ischemia-induced apoptotic neuronal cell death and thus is maybe useful agent for facilitating the recovery following ischemic cerebral injury.

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