Bee Venom Suppresses Ischemia-induced Increment of Apoptosis and Cell Proliferation in Hippocampal Dentate Gyrus

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Cerebral ischemia resulting from transient or permanent occlusion of cerebral arteries leads to neuronal cell death and eventually causes neurological impairments. Bee venom has been used for the treatment inflammatory disease. In the present study, the effects of bee venom on apoptosis and cell proliferation in the hippocampal dentate gyrus following transient global ischemia in gerbils were investigated using immunohistochemistry for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), caspase-3, and 5-bromo-2'-deoxyuridine (BrdU). It was shown that apoptotic cell death and cell proliferation in the hippocampal dentate gyrus were significantly increased following transient global ischemia in gerbils and that treatment of bee venom suppressed the ischemia-induced increase in apoptosis and cell proliferation in the dentate gyrus. The present results also showed that 1 mg/kg bee-venom treatment suppressed the ischemia-induced increasing apoptosis, cell proliferation, and COX-2 expression in the dentate gyrus. It is possible that the suppression of cell proliferation is due to the reduction of apoptotic cell death by treatment of bee venom. In the present study, bee venom was shown to prosses anti-apoptotic effect in ischemic brain disease, and this protective effect of bee venom against ischemia-induced neuronal cell death is closely associated with suppression on caspase-3 expression.

Key words : Transient global ischemia, bee venom, apoptotic cell death, cell proliferation, cycloogenase-2

Introduction

Cerebral ischemia results from reduction in cerebral blood flow due to transient or permanent occlusion of cerebral arteries[1,2]. Two distinct forms of neuronal cell death, apoptosis and necrosis, have been described based on morphological appearance. Apoptosis, also known as programmed cell death, is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations compared to necrosis[3,4]. It has been reported that apoptosis is a form of cell death that occurs in several pathological situations and that it constitutes part of a common mechanism in cell replacement, tissue remodeling, and removal of damaged cells[5]. Apoptosis is characterized by chromatin aggregation, cytoplasmic condensation, and formation of apoptotic bodies. The apoptotic bodies are engulfed and digested by surrounding cells[6-8]. Previous studies showed that delayed neuronal cell death following ischemic brain injury is different from early ischemic responses[9].

Caspase is known to mediate a crucial stage of the apoptotic process and is expressed in many mammalian cells. Caspases, which make up a family of cysteinyl proteases encompassing 14 members, are essential players in apoptotic cell death both as initiators (caspase-2, -8, -9, and -10) and executioners (caspase-3, -6, and -7)[10]. Important characteristic of apoptosis is the activation of caspase-3, the most widely studies member of the caspase family of cysteinyl proteases and one of the key mediators of apoptosis in mammalian cells. Caspase-3 plays a critical role during morphogenetic cell death in the mammalian brain[11].

The hippocampal formation generates new neurons throughout life in the mammals including humans[12-16]. Hippocampus is known to be implicated in memory acquisition and learning ability[12,17-19]. Previous studies have shown that cell proliferation in the hippocampus is increased by spatial learning, serotonin, estrogen, *N*-methyl-D-aspartate (NMDA) receptor antagonists, ischemia, seizure, and physical

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exercise, and inhibited by adrenal steroids, stress, and aging[18,20-22]. Generation of new cells in the adult brain is also enhanced by pathological events such as seizures and ischemic insults[23,24]. Such upregulation of cell proliferation occurring during pathological situations is thought to be a compensatory response to lesion-induced neuronal cell death in the brain[23]. Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins from arachidonic acid. Two isoforms of COX are known: . cyclooxygenase-1 (COX-1), a constitutively expressed enzyme responsible for the production of prostaglandins with general housekeeping functions[25,26] and cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase. COX-2 is closely associated to inflammation and control of cell growth[26,27]. Selective COX-2 inhibition has been proposed as a way to avoid these toxic effect[28].

Traditionally, bee venom has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and for the relief of pain[29,30]. Bee venom from the honeybee consists of melittin, phospholipase A2, apamin, adolapin, mast cell degranulating peptide, and several other bioactive substances[31]. Of these, melittin and phospholipase A2 are major components of bee venom and play an important role in the induction of irritation and allergic reaction. Phospholipase A2, a membrane-associated phospholipid, is a converting enzyme that is important in the production of arachidonic acid. Arachidonic acid is further metabolized into one of two pathways, prostaglandins (by cyclooxygenase) or leukotrienes (by lipooxygenase). Natural bee venom produces irritation when injected subcutaneously, while diluted bee venom, particularly injection into acupoints, can reduce chronic nociception and inflammation[30].

Materials and Methods

1. Animals and treatments

Adult male Mongolian gerbils (11 - 13 weeks of age) were used in this experiment. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health (NIH) and the Korean Academy of Medical Sciences. Each animal was housed under controlled temperature (22 ± 2℃) and lighting (08:00 h - 20:00 h) conditions with food and water made available ad libitum. Gerbils were randomly divided into five groups (n = 6 in each group): the sham-operation group, the ischemia-induction group, the ischemia-induction and 0.01 mg/kg bee-venom treatment group, the ischemia-induction and 0.1 mg/kg bee-venom treatment group, and the ischemia-induction and 1 mg/kg bee-venom treatment group.

2. Induction of transient global ischemia

To induce transient global ischemia in gerbils, a surgical procedure based on previously described experimental method[23] was performed. In brief, gerbils were anesthetized with 3% isoflurane in 20% O_2 77% N_2. Following bilateral neck incisions, both common carotid arteries (CCAs) were exposed and occluded with aneurysm clips for 5 min. The clips were then removed to restore cerebral blood flow. The rectal temperature was maintained at 37 0.5C with a heating lamp until the gerbils regained consciousness. After recovery, the animals were monitored for additional 2 h to prevent hypothermia. Animals of the sham-operation groups were treated identically, except that the CCAs were not occluded after the neck incisions.

3. Drug treatment

5-Bromo-2'-deoxyuridine (BrdU) (50 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) was given intraperitoneally to all animals 1 h before the bee-venom treatment once a day for 10 days.

Bee venom of Apis mellifera (Sigma Chemical Co., St. Louis, MO, USA) at doses of 0.01, 0.1 and 1 mg/kg was dissolved in physiological saline solution (100 μl) and subcutaneously administered into Zusanli (ST36) acupoint located 5 mm lower and lateral to the anterior tubercle of the tibia in the knee joint of the hind limb. Zusanli acupoint is one of the most widely used acupoints prossesses analgesic, spasmolytic, and homeostatic actions and has been used in various brain disorders, especially for the treatment of cerebrovascular disease in humans and animals[32-36]. Rats of the ischemia-induction groups received respective doses of 0.01, 0.1, and 1 mg/kg bee venom into the Zusanli acupoint for 10 consecutive days from the starting of experiment. All animals were sacrificed 1 h after bee venom injection on the 10th day.

4. Tissue preparation

To begin the sacrificial process, animals were fully anesthetized using Zoletil 50 (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (PFA) in 100 mM phosphate buffer (PB) at pH 7.4. The brains were removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 ㎛ thickness were made using a freezing microtome (Leica, Nussloch, Germany).

5. Caspase-3 immunohistochemistry

For visualization of caspase-3 expression, caspase-3 immuno-

histochemistry was performed. Sections were incubated overnight with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then for another 1 h with biotinylated mouse secondary antibody. Bound secondary antibody was then amplified with Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in 50 mM Tris-buffer (pH 7.6) 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.

6. BrdU immunohistochemistry

For detection of newly generated cells in the dentate gyrus, BrdU-specific immunohistochemistry was performed. Sections were first permeabilized by incubation in 0.5% Triton X-100 in PBS for 20 min, then pretreated in 50% formamide-2 x standard saline citrate (SSC) at 65C for 2 h, denaturated in 2 N HCl at 37C for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4℃ 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, Burlingame, CA, USA). Then the sections were incubated for another 1 h with avidin-peroxidase complex (1:100; Vector Laboratories, Burlingame, CA, USA). 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% H_2O_2 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 .

7. Cyclooxygenase-1 and cyclooxygenase-2 immunohistochemistry

Sections were incubated in PBS for 10 min and washed three times, again with PBS. The sections were then incubated in 1% H_2O_2 for 30 min. Next, the sections were incubated overnight with rabbit anti-COX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 for visualization of COX-1 expression or with rabbit anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 for visualization of COX-2 expression. The sections were then incubated for 1 h with biotinylated anti-goat secondary antibody (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.02% 3,3'-DAB and 0.03% H_2O_2 in 50 mM Tris-buffer (pH 7.6) for approximately 3 min. The sections were then mounted on gelatin-coated glass slides.

8. Data analysis

The numbers of COX-1-positive, COX-2-positive, and caspase-3-positive cells in the hippocampal dentate gyrus and the number of BrdU-positive cells in the subgranular layer of dentate gyrus were counted hemilaterally in every eighth section throughout the entire extent of the dentate gyrus at 400 magnification. The area of the granular layer of dentate gyrus was traced using Image-ProPlus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA) at 40 magnification. The numbers of COX-1-positive, COX-2-positive, caspase-3-positive, and BrdU-positive cells were expressed as mean number of cells per mm2 of the cross sectional area of the granular layer of the dentate gyrus. Data was expressed as a mean ± standard error mean (S.E.M). For comparison between groups, one-way ANOVA and Duncan's post-hoc test were performed with $P < 0.05$ as an indication of statistical significance.

Results

1. Effects of bee venom on caspase-3-positive cells in dentate gyrus

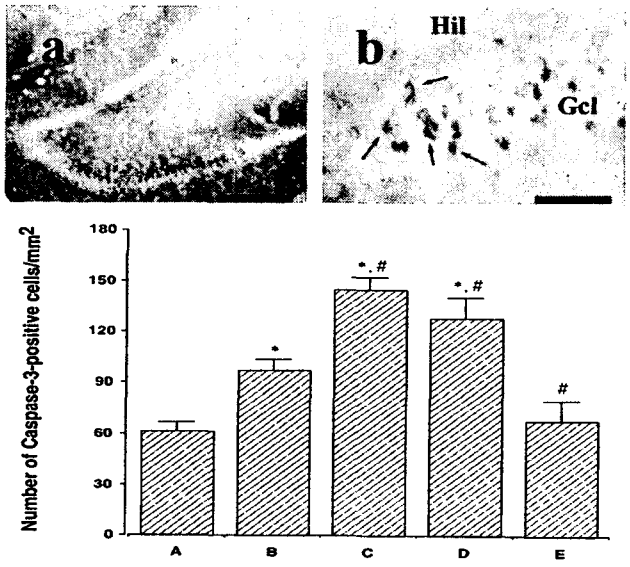

Fig. 1. Effect of bee venom on caspase-3 expression in the dentate gyrus after transient global ischemia. Upper: A photomicrograph of caspase-3-positive cells. Sections were stained for caspase-3-positive cells. (a) and (b) caspase-3 labeled nuclei (black arrows). Gcl, granular cell layer; Hil, hilus. Scale bar represents 100 ㎛ (a) and 25 ㎛ (b). Lower: Mean number of caspase-3-positive cells in each group. A, sham-operation group; B, ischemia-induction group; C, ischemia-induction and 0.01 mg/kg bee venom treatment group; D, ischemia-induction and 0.1 mg/kg bee venom treatment group; E, ischemia-induction and 1 mg/kg bee venom treatment group. Values are represented as mean ± S.E.M. * represents $P < 0.05$ compared to the sham-operation group. # represents $P < 0.05$ compared to the ischemia-induction group.

A photomicrograph of caspase-3-positive cells in the dentate gyrus is shown in Fig. 1. The number of caspase-3-positive cells was 61.74 ± 4.95/mm^2 in the sham-operation group, 97.38 ± 6.29/mm^2 in the ischemia-induction group, 145.03 ± 7.48/mm^2 in the ischemia-induction and 0.01 mg/kg bee venom treatment group, 128.01 ± 12.72/mm^2 in the ischemia-induction and 0.1 mg/kg bee venom treatment group, and 68.21 ± 12.10/mm^2 the ischemia-induction and 1 mg/kg bee venom treatment group.

Caspase-3 expression was increased by ischemia and bee venom at the dose of 0.01 mg/kg and 0.1 mg/kg enhanced ischemia-induced increasing of caspase-3 expression, and 1 mg/kg bee venom treatment suppressed ischemia-induced increment of caspase-3 expression.

2. Effect of bee venom on cell proliferation in dentate gyrus

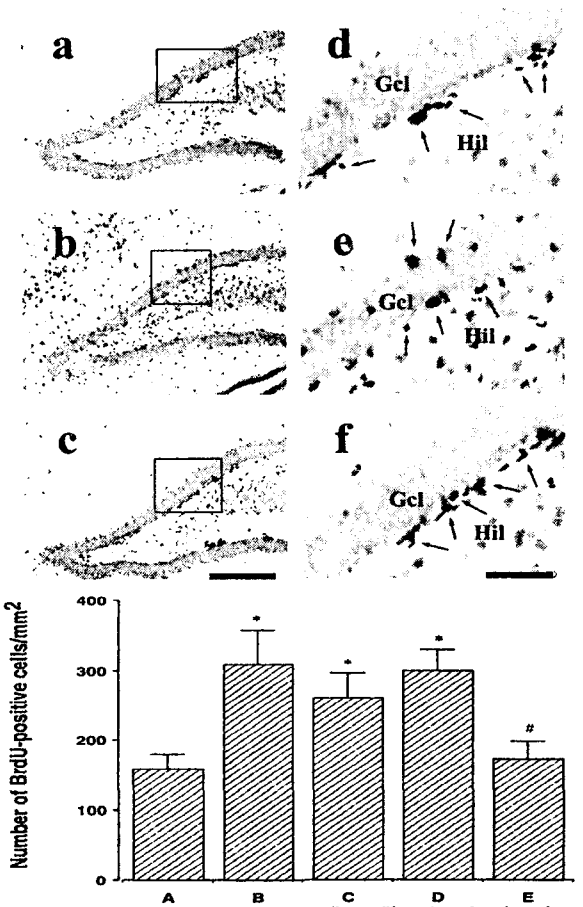

Fig. 2. Effect of bee venom on cell proliferation in the dentate gyrus after transient global ischemia. Upper: A photomicrograph of BrdU-positive cells. Sections were stained for BrdU (black arrows) and neuronal nuclei (NeuN) (brown). (a) and (d), sham-operation group; (b) and (e), ischemia-induction group; (c) and (f), ischemia-induction and 1 mg/kg bee venom treatment group. GCL, granule cell layer; Hil, hilus. Scale bar in (a), (b), and (c) represents 100 ㎛ and scale bar in (d), (e), and (f) represents 25 ㎛. Lower: Mean number BrdU-positive cells in each group. A, sham-operation group; B, ischemia-induction group; C, ischemia-induction and 0.01 mg/kg bee venom treatment group; D, ischemia-induction and 0.1 mg/kg bee venom treatment group; E, ischemia-induction and 1 mg/kg bee venom treatment group. Values are represented as mean ± S.E.M. * represents P < 0.05 compared to the sham-operation group. # represents P < 0.05 compared to the ischemia-induction group.

A photomicrograph of BrdU-positive cells in the dentate gyrus is shown in Fig. 2. The number of BrdU-positive cells was 159.50 ± 20.15/mm^2 in the sham-operation group, 309.41 ± 47.34/mm^2 in the ischemia-induction group, 260.47 ± 35.06/mm^2 in the ischemia-induction and 0.01 mg/kg bee venom treatment group, 300.19 ± 28.97/mm^2 in the ischemia-induction and 0.1 mg/kg bee venom treatment group, and to 173.42 ± 24.78/mm^2 the ischemia-induction and 1 mg/kg bee venom treatment group.

A significant increment in cell proliferation was observed following transient global ischemia, and 1 mg/kg bee venom treatment suppressed the ischemia-induced increment of cell proliferation.

3. Effect of bee venom on the cyclooxygenase-1 expression in the dentate gyrus

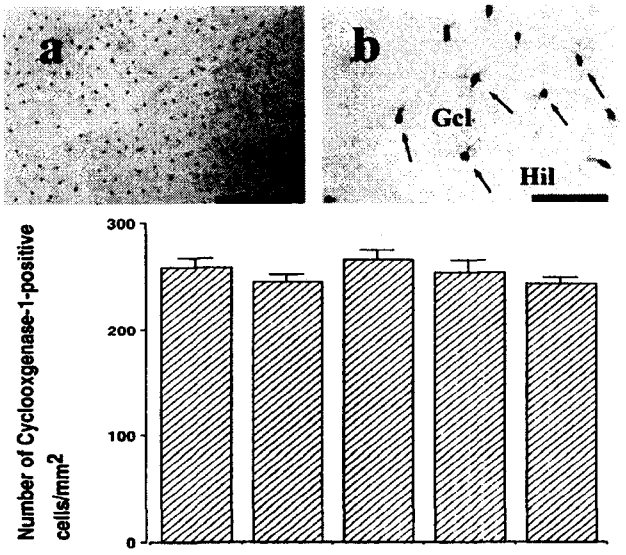

Fig. 3. Effect of bee venom on COX-1 expression in the dentate gyrus after transient global ischemia. Upper: A photomicrograph of cyclooxygenase-1-positive cells. Sections were stained for COX-1-positive cells. (a) and (b) COX-1 labeled nuclei (black arrows). Gcl, granular cell layer; Hil, hilus. Scale bar represents 100 ㎛ (a) and 25 ㎛ (b). Lower: Mean number of cyclooxygenase-1-positive cells in each group. A, sham-operation group; B, ischemia-induction group; C, ischemia-induction and 0.01 mg/kg bee venom treatment group; D, ischemia-induction and 0.1 mg/kg bee venom treatment group; E, ischemia-induction and 1 mg/kg bee venom treatment group. Values are represented as mean ± S.E.M.

A photomicrograph of COX-1-positive cells is shown in Fig. 3. The number of COX-1-positive cells was 258.51 ± 8.66/mm^2 in the sham-operation group, 245.59 ± 6.56/mm^2 in the ischemia-induction group, 266.28 ± 8.42/mm^2 in the ischemia-induction and 0.01 mg/kg bee venom treatment group, 254.00 ± 11.47/mm^2 in the ischemia-induction and 0.1 mg/kg bee venom treatment group, and 243.32±6.18/mm^2 the ischemia-induction and 1 mg/kg bee venom treatment group.

Ischemia and bee venom exerted no significant effect on the COX-1 expression in the dentate gyrus.

4. Effect of bee venom on the cyclooxygenase-2 expression in the dentate gyrus

A photomicrograph of COX-2-positive cells is shown in Fig. 4. The number of COX-2-positive cells was 93.94 ± 8.77/mm^2 in the sham-operation group, 197.30 ± 10.91/mm^2 in the ischemia-induction group, 221.40 ± 10.91/mm^2 in the ischemia-induction and 0.01 mg/kg bee venom treatment group, 207.52 ± 8.13/mm^2 in the ischemia-induction and 0.1 mg/kg bee venom treatment group, and 169.08 ± 5.87/mm^2 the ischemia-induction and 1 mg/kg bee venom treatment group.

COX-2 expression in the dentate gyrus was increased by transient global ischemia and 1 mg/kg bee venom treatment suppressed ischemia-induced increment of COX-2 expression.

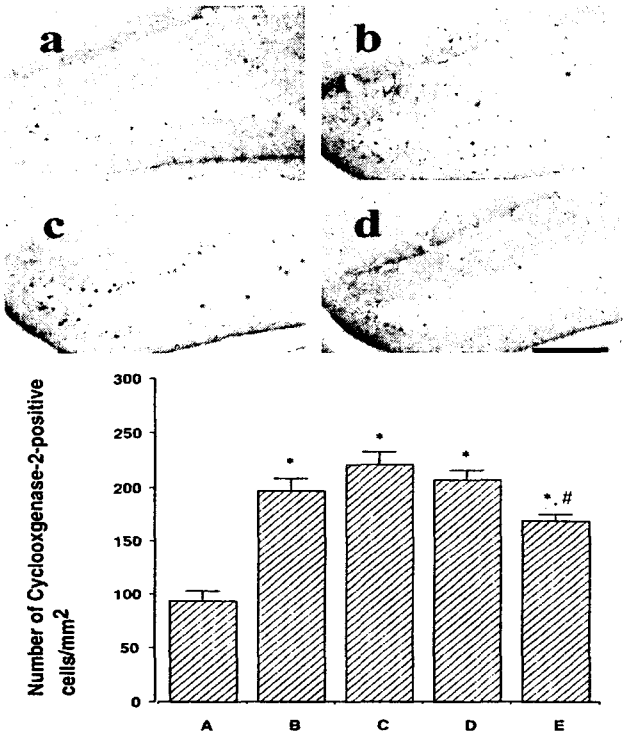

Fig. 4. Effect of bee venom on COX-2 expression in the dentate gyrus after transient global ischemia. Upper: A photomicrograph of cyclooxygenase-2-positive cells. (a), sham-operation group; (b), ischemia-induction group; (c), ischemia-induction and 0.01 mg/kg bee venom treatment group; (d), ischemia-induction and 1 mg/kg bee venom treatment group. Scale bar represents 100 ㎛. Lower: Mean number of cyclooxygenase-2-positive cells in each group. A, sham-operation group; B, ischemia-induction group; C, ischemia-induction and 0.01 mg/kg bee venom treatment group; D, ischemia-induction and 0.1 mg/kg bee venom treatment group; E, ischemia-induction and 1 mg/kg bee venom treatment group. Values are represented as mean±S.E.M. * represents P<0.05 compared to the sham- operation group. # represents P < 0.05 compared to the ischemia-induction group.

Discussion

The present study was designed to determine the effects of bee venom on cell proliferation and apoptosis in the hippocampal dentate gyrus region in relation with COX expression after transient global ischemia in gerbils.

Caspases, a family of cysteine protease, play a central role in the control of apoptosis[37,38]. Fourteen distinct caspases are divided into initiator or effector based on their role and position in apoptotic cascades and involved in the cellular regulation of programmed cell death[9]. They mediate morphological and biochemical features of apoptosis, including structural dismantling of cell bodies and nuclei, fragmentation of genomic DNA, destruction of regulatory proteins, and propagation of other pro-apoptotic molecules. Caspases have received a great deal of attention due to their involvement in various types of cell death under normal development and pathological conditions[39-41]. Of these, caspase-3 is the most abundant caspase and serves as a downstream executioner. In addition, activation of caspases was reported in apoptosis following ischemia[42,43]. In the present results, increased caspased-3 expression was observed in the dentate gyrus of transient global ischemic gerbils. Liu et al.[23] reported that cell proliferation in the dentate gyrus of gerbils is significantly increased after ischemia. It is generally believed that ischemia-induced increment in cell proliferation is a compensatory response to excessive apoptotic cell death. In the present results, increased cell proliferation was observed in the dentate gyrus of in transient global ischemic gerbils.

Nam et al.[44] suggested that pharmacological activities of the bee venom on anti-inflammatory process include the inhibition of COX-2 expression and the blocking of pro-inflammatory cytokines (TNF-alpha, and IL-1beta) production. Bee venom showed dose-dependent inhibitory effects on COX-2 activity, but did not inhibit COX-1 activity, in this study. Previous study showed that long-term treatment with bee venom at a dose of 1 mg/kg/day produces a significant anti-nociceptive and anti-inflammatory effect on the Freund's complete adjuvant-induced arthritis in rats[29]. In the present results, enhanced COX-2 expression was observed in the dentate gyrus of transient global ischemic gerbils.

The present results showed that 1 mg/kg bee venom treatment suppressed the ischemia-induced increasing of apoptosis, cell proliferation, and COX-2 expression in the dentate gyrus. It is possible that the suppression of cell proliferation is due to the reduction of apoptotic cell death by bee venom. Present study revealed that bee venom prossess anti-apoptotic effect in ischemic brain disease, and this protective effect of bee venom against ischemia-induced neuronal cell death is closely associated with suppression on caspase-3 expression.

Acknowledgments

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