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원 저

Effect of bee venom on cell proliferation and cyclooxygenase 2 expression in the dentate gyrus of mice with acetic acid induced hyperalgesia

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국문초록

세포 증식과 COX 2 발현에 미치는 봉독의 효과

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연구 배경 및 목적 : 봉독은 항염증 등의 효과를 가지고 있다고 알려져 있지만 세포 증식과 관련된 봉독의 효과에 대한 고찰을 위해 본 硏究에서는, 蜂毒 藥鍼 刺戟이 아세트산 誘發 痛覺 過敏症을 가진 쥐의 齒狀回에서의 細胞 增植과 COX 2 發現에 미치는 影響에 대해 알아보고자 하였다.

실험 방법 : 대조군, 아세트산 처치군, 아세트산 0.1mg/kg 봉독 처치군, 아세트산 1mg/kg 봉독 처치군(n=5 in each group)의 네 군으로 나누고 해당 군에 일벌 蜂毒(Sigma Chemical Co., St. Louis, MO, USA)을 양측 족삼리 경혈(ST36)에 주입시키고 30분 후 아세트산(100% acetic acid 1% 용액의 0.5ml)을 복강내로 주입하여 복부 긴장 횟수를 세었으며, BrdU 양성, COX 2 양성 세포수를 면역 화학 조직법을 수행하여 세어 보았다.

결과 : 아세트산 처치군에서는 대조군에 비해 5 bromo 2' deoxyuridine 양성 세포의 수는 減少되며, 齒狀回에서의 COX 2의 發現은 增强되는 것으로 보여졌다. 蜂毒 注入은 아세트산 誘發 腹部 緊張 횟수과 齒狀回에서의 COX 2 發現을 抑制하여, 齒狀回에서의 細胞 增植을 增加시켰다.

결론 : 이번 結果에서 보면, 齒狀回에서의 COX 2의 發現은 細胞 增植 抑制와 關聯되며 蜂毒은 COX 2 發現 抑制를 통해 齒狀回에서의 새로운 細胞 形成을 增加시킨다는 것을 알 수 있다.

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I. Introduction

Bee venom therapy is an acupuncture therapy applying the pharmacological action of stimulation and bee venom to the body. It is executed by pricking disease associated sites or particular acupuncture points with bee venom extracted with electricity or from honeybee directly[1)~3)].

Traditionally, bee venom has been used for the relief of pain and the treatment of inflammatory diseases such as rheumatoid arthritis[4),5)].

Bee venom therapy has been processed and reported in many experimental studies, with regard to its effect in pain alleviation, anti inflammation, removal of fever[6)], anti convulsion, suppression of tumor and immunity strengthening effect etc.[7)].

Hippocampal neurogenesis has been observed in various adult animals, including humans[8),9)]. Newly generated cells are thought to play a role in learning, memory, and brain repair[10)~12)], however, the cellular mechanisms underlying neurogenesis remain unknown. Previous studies have shown that several factors such as glucocorticoids, estrogen, N methyl D aspartate(NMDA) receptor antagonists, growth factors, serotonin, aging, seizures, and environmental stimuli influence the proliferation of granule cell precursors and/or neurogenesis in the adult dentate gyrus[9)~11),13),14)].

Cyclooxygenase(COX), an enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, has two isoforms ; COX 1 and COX 2. While COX 1 is a constitutively expressed form required for normal physiologic functions, COX 2 is inducible in response to cytokines, growth factors, or other inflammatory stimuli[15)~17)]. Recently, Kumihashi et al.[18)] reported that the level of COX has a bearing on neurogenesis in ischemic gerbils, and it has been showed that COX 2 may be involved in neruodegenerative disorders such as Alzheimer's disease and ischemic neuropathy[19),20)].

Bee venom is known to possess anti inflammatory effects[21)] similar to those seen for non steroidal anti inflammatory drugs(NSAIDs). However, the effects of bee venom at jogsamni(ST36) in relation to cell proliferation and expression of COX 2 in the dentate gyrus have not yet been reported. In the present study, the effects of bee venom on new cell formation and expression of COX 2 in the dentate gyrus are investigated via immuno-

histochemistry.

II. Materials and Methods

1. Animlas and Treatment

Male C57/B6 mice weighing 25g(corresponding to 10 weeks of age) were used for the experiment. The experimental procedures were performed in accordance with the animal care guidelines of NIH and the Korean Academy of Medical Sciences. Each animal was housed at a controlled temperature(20±2℃) and maintained under light dark cycles, each consisting of 12 h of light and 12 h of darkness(lights on from 07 : 00 h to 19 : 00 h).

Animals were divided into four groups : the control group, the acetic acid treated group, the acetic acid and 0.1mg/kg bee venom treated group, and the acetic acid and 1mg/kg bee venom treated group(n=5 in each group). Mice of the acetic acid treated groups were injected intraperitoneally with acetic acid(0.5 ml of 1% solution of 100% acetic acid). The numbers of abdominal stretches during two consecutive periods of 30 min each were counted by two experienced investigators uninformed of the experimental treatment given to each rat. Mice of the bee venom treated groups were injected with bee venom(Sigma Chemical Co., St. Louis, MO, USA) at the respective doses, 30 min before acetic acid injection. The Jogsamni acupoint(ST36)[22)-24)], located 5mm lateral and distal to the anterior tubercle of the tibia, was chosen as the injection site for bee venom due to the traditional use of the Jogsamni acupoint for the relief of pain[7)]. All mice received 50 mg/kg of 5 bromo 2' deoxyuridine(BrdU ; Sigma Chemical Co., St. Louis, MO, USA) intraperitoneally 30 min before acetic acid injection. Animals were sacrificed immediately after counting the number of stretches.

2. Tissue Preparation

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

3. BrdU and COX 2 Immunohistochemistry

For visualization of cell birth in the dentate gyrus, BrdU specific immunohistochemistry was performed[25)]. An average of eight sections within the dorsal hippocampal region spanning from Bregma 3.30 mm to 4.16 mm were selected from each brain. The sections were first pretreated by incubating in 50% forma-

mide 2 x standard saline citrate at 65℃ for 2 h, denaturing in 2 N HCl at 37℃ for 30 min, and rinsing twice in 100 mM sodium borate (pH 8.5). After the pretreatment, the sections were incubated overnight at room temperature with BrdU specific mouse monoclonal antibody (1 : 600) (Boehringer Mannheim, Mannheim, Germany). The sections were washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1 : 100) (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated for another 1 h with an avidin biotin horseradish peroxidase complex (1 : 100) (Vector Laboratories, Burlingame, CA, USA). For staining, the sections were incubated with 0.02% 3,3' diaminobenzidine containing nickel chloride (40 mg/ml) (Nickel DAB) and 0.03% hydrogen peroxide in 50 mM Tris HCl (pH 7.6) for 5 min. Finally, the sections were mounted onto gelatinized glass slides. The slides were air dried overnight at room temperature, and cover slides were mounted using Permount®.

For visualization of COX 2 expression via COX 2 immunohistochemistry, free floating tissue sections were first washed twice in 50 mM PBS and were then permeabilized by incubation in 0.2% Triton X 100 for 30 min. After washing twice with PBS, sections were incubated overnight with goat anti COX 2 antibody (DiaSorin, Stillwater, MN, USA) at a dilution of 1 : 1000. Sections were washed twice in PBS and incubated for 1 h with biotinylated anti goat antibody (1 : 200). 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 0.05% diaminobenzidine. The sections were mounted onto gelatinized glass slides, air dried, and cover slides were mounted using Permount®.

4. Data Analysis

The area of the dentate gyrus was measured using an image analyzer (Multiscan, Fullerton, CA, USA). The numbers of BrdU positive and COX 2 positive cells were counted hemilaterally as cells per mm^2 of cross sectional area of the granular layer of the dentate gyrus. Data was analyzed using SPSS (version 7.5) by one way analysis of variance (ANOVA) followed by Scheffe's Post hoc test and results were expressed as mean± standard error mean (S.E.M.). The level of statistically significant differences was defined as $P<0.05$.

Ⅲ. Results

1. Effect of bee venom on acetic acid induced abdominal stretches

The numbers of abdominal stretches in the experimental groups are summarized in <Table 1>. In the results, bee venom treatment inhibited acetic acid induced abdominal stretch reflex significantly.

Table 1. The total numbers of abdominal stretches of each group.

Group	The number of abdominal stretches during the first 30min.	The number of abdominal stretches during the latter 30min.
Control	0	0
Acetic acid treated	33.00±5.05[a]	7.05±1.51[a]
Acetic acid and 0.1mg/ml bee venom treated	19.00±3.84[a,b]	2.50±0.81[b]
Acetic acid and 1mg/ml bee venom treated	20.00±0.74[a,b]	0[b]

Values represent mean±S.E.M.

[a] represents $P<0.05$ compared to the control group.

[b] represents $P<0.05$ compared to the acetic acid treated group.

Data was analyzed using SPSS(version 7.5) by one way analysis of variance(ANOVA) followed by S

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

The mean number of BrdU positive cells in the dentate gyrus was 52.50±3.20/mm^2 in the control group, 31.00±2.10/mm^2 in the acetic acid treated group, 69.67±4.58/mm^2 in the acetic acid and 0.1mg/kg bee venom treated group and 73.00±7.08/mm^2 in the acetic acid and 1mg/kg bee venom treated group<Fig. 1>.

3. Effect of bee venom on COX 2 expression in hippocampal dentate gyrus

The mean number of COX 2 positive cells in the dentate gyrus was 41.00±2.34/mm^2 inthe control group, 130.00±12.01/mm^2 in the acetic acid treated group,61.41±5.73/mm^2 in

Fig. 1. Effect of bee venom on cell proliferation.

Above : Photomicrographs of 5 bromo 2' deoxyuridine(BrdU) positive cells in each group. Sections were stained for BrdU positive cells(black). Scale bar represents 50㎛.

Below : Mean number of BrdU positive cells in the subgranular layer of the dentate gyrus in each group. Values are represented as mean±S.E.M.

[a] represents $P<0.05$ compared to the control group.

[b] represents $P<0.05$ compared to the acetic acid treated group.

A, Control group ;

B, Acetic acid treated group ;

C, Acetic acid and 0.1mg/kg bee venom treated group ;

D, Acetic acid and 1mg/kg bee venom treated group.

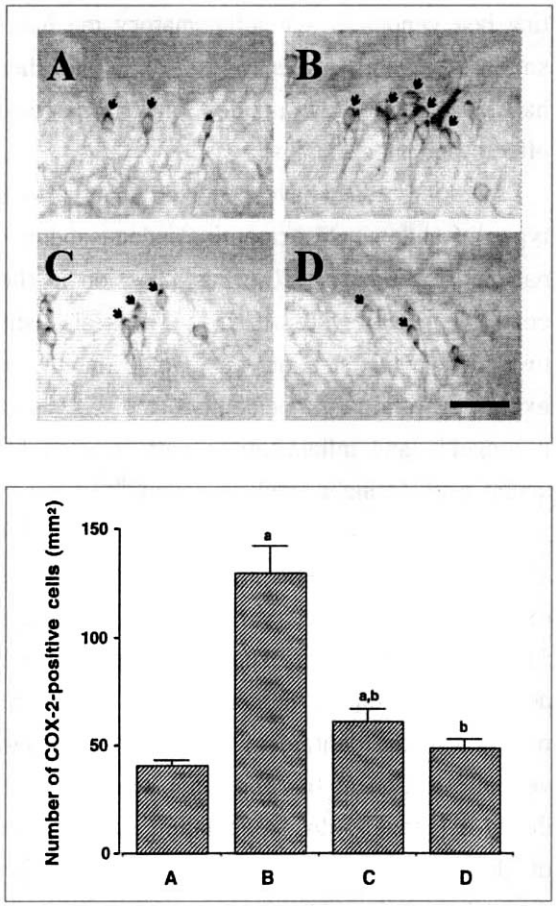

Fig. 2. Effect of bee venom on cyclooxygenase(COX) 2 expressions.

Above : Photomicrographs of COX 2 positive cells in each group. Sections were stained for COX 2 positive cells(reddish brown). Scale bar represents 25㎛.

Below : Mean number of COX 2 positive cells in the granular layer of the dentate gyrus in each group. Values are represented as mean±S.E.M.

[a] represents $P<0.05$ compared to the control group.

[b] represents $P<0.05$ compared to the acetic acid treated group.

A, Control group ;

B, Acetic acid treated group ;

C, Acetic acid and 0.1mg/kg bee venom treated group ;

D, Acetic acid and 1mg/kg bee venom treated group.

the acetic acid and 0.1mg/kg bee venom treated group and 49.25±3.89/mm^2 in the acetic acid and 1 mg/kg bee venom treated group <Fig. 2>.

Ⅳ. Discussion

Bee venom therapy is an acupoint injection therapy that controls physical functions and enhances the self recovering and the balance of the body. It is achieved through mechanical stimulation and specific bee venom impetus of pricking directly with honeybee needles or injecting bee venom extracted into abnormal section or disease associated acupuncture point ; namely acupoint, the extraordinary points, new acupoint, pressure pain point and touchable skin positive response point. Bee venom therapy might be a therapy that promotes the normalization of an autonomic nerve and the recuperation of health by pricking the region of congestion and pain.

The effects of bee venom includes scientific effects such as powerful sterilization, hemolysis, subcutaneous permeation, promotion of blood circulation, relief of pain, regulation of the autonomic nervous system and its anti inflammatory effects by applying sharp pain to the congestion site. The acupuncture effects and that of acupuncture and moxibustion is a therapy acceleratig normalization of the autonomic nervous system and recovery of health.

It has been suggested that the effect of

bee venom therapy is superior to that of general acupuncture therapy, in that it has not only physical but also chemical actions[26]. It might be said that these various effects and indications for the use of bee venom originate from the procedures of general acupuncture therapy[6),27),28)]. The four effects of bee venom produce remarkable results. The first effect is the acupuncture action of stimulating the nerve by applying acupuncture to an acupoint. Another is the continuous moxibustion operation by producing heat for several days there. The third is the injection effect, which includes hemolysis, sterilization, anti inflammation and alleviation of pain action by penetrating into the interior of body. The fourth is the edema effect, where the affected part becomes swollen, vessels get dilated and both blood flow and blood cell increase rapidly[18)].

Bee venom can be applied in cases of stiff shoulders, frozen shoulders, back pain, sprained back pain, myalgesia, migraine, numbness, knee pain, the common cold, sty, asthenopia, neuralgia, trigeminal neuralgia, intercostal neuralgia, sprain, sciatic neuralgia, stomatitis, ophthalmalgia from stiff shoulders, meralgia by a menopausal disorder, chill menstrual pain, asthma, rheumatism, hay fever (disorder of eye, nose, throat etc.), gastric ulcers, hypertension, insomnia, diabetes mellitus etc.[1)].

Also, according to previous studies, the components of bee venom's lowers symptoms of acute, chronic arthritis and edema followed by inflammation[29),30)]. Many studies investigating bee venom' s anti inflammatory mechanisms are currently underway[31),32)]. On the other hand, there are few studies of its analgesic effects and mechanisms.

Previous studies have reported that various types of abdominal nociception induce abdominal pain by inducing COX 2 expression in the central nervous system(CNS). It was also shown that administration of COX 2 inhibitors exerts systemic antinociceptive action against neurogenic and inflammatory pain induced by acetic acid, formalin, and capsaicin[33),34)].

From the results, it was demonstrated that administration of acetic acid increases COX 2 expression, while bee venom injection at the Jogsamni acupoint was shown to suppress the acetic acid induced increase in COX 2 expression in the dentate gyrus. In addition, bee venom injection at the Jogsamni acupoint produced a time and dose dependent suppression of the frequency of abdominal stretches induced by acetic acid.

The Jogsamni acupoint used in this study is the Hab To Acupoint of the Stomach Meridian and has a "character of harmony of Gi Hyul, run of meridian". It shows analgesic effects on various gastrointestinal and systemic disorders and lower limbs diseases[23)]. Kwon et al. reported that administration of bee venom at the acupoints Jogsamni and Zhongwan produces a more potent antinociceptive effect than at a non acupoint in mice with adjuvant induced arthritis and acetic acid induced visceral pain[29),30)].

Also, in the treatment of knee arthritis, bee

venom is more effective than general acupuncture[26], while stimulating action shows that injection into an acupoint is more effective than a non acupoint[35].

In adult rats, neuronal precursors are known to reside in the subgranular zone of the dentate gyrus, where they proliferate and migrate continuously into the granular cell layer and differentiate into mature neurons demonstrating morphological and biochemical features observed in the surrounding neurons[10]. These newly formed neurons have been shown to perform an important role in memory formation[12]. The neurogenesis in the dentate gyrus is known to be enhanced by environmental factors and stimuli such as physical activity[9], serotonin[11], estrogen[14], and antidepressants[36,37]. On the other hand, neurodegenerative conditions and stressful experiences, including physical and psychosocial stress, have been shown to suppress the proliferation of hippocampal granule cells in the dentate gyrus of mammals[13,36].

It has been suggested that anti inflammatory drugs such as NSAIDs possess protective effect in neurodegenerative disorders by inhibiting COX 2 expression[20]. Recently, Kumihashi et al.[38] reported that the level of COX 2 has an effect on neurogenesis and that COX inhibitor possesses a protective effect against ischemia induced cell death. In the present results, acetic acid administration decreased new cell formation, while bee venom injection at the Jogsamni acupoint increased new cell formation in the dentate gyrus of mice significantly. Also, Bee venom treatment was thus shown to suppress the acetic acid induced increase of COX 2 expression in the dentate gyrus. Based on the results of the present study, it can be suggested that expression of COX 2 in the dentate gyrus has a negative effect on cell proliferation and that bee venom increases new cell formation in the dentate gyrus via inhibition of COX 2 expression.

V. Conclusion

In this study, the effects of bee venom on cell proliferation and expression of cyclooxygenase(COX) 2 in the dentate gyrus of mice with acetic acid induced hyperalgesia were investigated. In the acetic acid treated group, the number of 5 bromo 2' deoxyuridine positive cells was shown to be decreased and the expression of COX 2 in the dentate gyrus enhanced compared to the control group. Bee venom acupoint injection resulted in suppression of the frequency of acetic acid induced abdominal stretches and COX 2 expression in the dentate gyrus and an increase in cell proliferation in the dentate gyrus. From the present results, it can be suggested that COX 2 expression in the dentate gyrus is implicated in inhibition of cell proliferation and that bee venom increases new cell formation in the dentate gyrus, probably by inhibition of COX 2 expression.

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