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원 저

Scavenging Effect of Bee Venom for Acua-acupuncture against Nitric Oxide

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초 록

봉독약침액의 Nitric Oxide에 대한 소거 효과

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목 적 : 본 연구는 최근 임상에서 많이 사용하는 봉독약침액의 nitric oxide (NO)에 대한 소거 효과를 분석하기 위하여 실행되었다.

방 법 : 양성대조군으로 비타민과 실험군으로 국산 봉독과 미국산 봉독에 NO를 분비하는 S-nitroso-N-acetylpenicillamine (SNAP)을 투여한 후 NO의 농도를 540 nm 파장의 자외선 흡수량을 측정하여 평가하였다.

결 과 : 국산 봉독은 100 ㎍/㎖에서 SNAP처리 1.5, 3, 6 및 12 시간에 대조군 (100%)과 비교하여 각각 86.7±1.5, 103.8±1.9, 88.3±2.9 및 85.6±3.2%의 잔류 NO 농도를 보였고, 1.5, 6 및 12시간에서 유의한 NO 소거 효과를 나타냈다. 미국산 봉독에서는 각각 74.0±2.2, 66.5±10.9, 86.1±2.8 및 59.1±3.8%로 모든 시간에서 유의한 결과를 보였다.

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결 론 : 이상의 봉독의 NO 소거효과는 봉독 약침액이 임상에서 동통, 종창 등에 치료에 사용하는 작용기전의 하나로 사료된다.

Key Words : Bee Venom, Acua-acupuncture, Nitric Oxide (NO), S-nitroso-N- acetylpenicillamine (SNAP)

I. INTRODUCTION

Free radicals from both exogenous and endogenous sources are known to damage tissue components. Since tissue damage by free radical increases with age, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO), and superoxides have been implicated in the pathogenesis of diseases associated with aging, such as cardiovascular diseases, cancers, arthritis and neurodegenerative diseases[1,2].

In the central nervous system NO is thought to play important roles in neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity, and regulation of gene expression. However, excessive production of NO following a pathologic insult is thought to lead to neurotoxicity. That neurotoxicity may related the pathogenesis of neurodegenerative disorders, including stroke, Parkinson's Disease and dementia[3].

Bee venom therapy has been used for many years in traditional oriental medicine. First record of bee venom treatment dates back to 168 B.C.[4]. In modern oriental medicine bee venom therapy is being used for acua-acupuncture to relieve pain and to cure inflammatory disease such as rheumatoid arthritis, osteoarthritis, and gout. The pharmacological properties of bee venom has been studied[5,6], and the antinociceptive and anti-inflammatory effect of bee venom is being demonstrated[7]. However, few reports have focused on the antioxidant effects of bee venom. The authors have previously examined the effects of bee venom in an attempt to elucidate the underlying mechanism of its action. The purpose of this present study is to find out whether bee venom possesses a scavenger effect against NO.

II. MATERIALS AND METHODS

1. Bee Venom Extract Preparation

Whole bee venom (Apis mellifera) of Korean bees (BV-K) was obtained by stimulating the bee sac with an electromagnetic extractor and dehydrating the obtained fluid. 0.1 g of BV-K was diluted with 99.9 ml of distilled water to make 1 ㎎/㎖ of BV-K. The liquid was then sterilized by heating at 121°C for 20 minutes in an autoclave. The resulting solution was further diluted to final concentrations of 1, 10 and 100 ㎍/㎖. Whole

bee venom from USA. (BV-U) was purchased from the Monmouth Pain Institute, at an initial concentration of 1 ㎎/㎖. It was further diluted to the final concentrations of 1, 10 and 100 ㎍/㎖.

2. Chemicals and Apparatus

Sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride for 50 mM phosphate buffer saline and N-(1-napthyl) ethylenediamine dihydrochloride, sulfanilamide, and H_3PO_4 for the Griess reagent were also purchased from Sigma (St. Louis, USA). An ELISA microplate reader (Molecular Device, USA) was used to measure absorbances at the wavelength of 540 nm.

3. Standard Curve for NO_2-Concentration

First, standard solutions with concentrations of 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 uM were made with sodium nitrite ($NaNO_2$). The absorbances of the solutions at 540 nm following the addition of the Griess reagent were measured using a microplate reader. A standard curve was then drawn using these known concentrations of sodium nitrite and the corresponding absorbances. The formula relating concentration and absorbance was then derived.

4. SNAP Preparation

S-nitroso-N-acetylpenicillamine(SNAP) was prepared as previously reported[8]. In brief, 10 mM N-acetylpenicillamine in 100% methanol/1 N HCl solution was made. 20 mM $NaNO_2$ was slowly added above mixture with vigorous stirring at 25℃. After approximately 20 minutes, SNAP was appeared with deep green color.

5. NO_2-Concentration Following Addition of SNAP Alone

To determine the NO_2^- concentration following the addition of SNAP, SNAP solutions at concentrations of 0.05, 0.125, 0.25, 0.5, 1, 2, and 4 mM were left in a humidified incubator containing 5% CO_2 and 95% O_2 at 37℃, and the absorbance of each solution was measured after 1.5, 3, 6 and 12 hours. To measure the absorbance the Griess reagent was added to each solution. Nitrite concentration was obtained by measuring the absorbance at 540 nm using a microplate reader.

6. Measurement of NO Scavenging Effect

Nitrite measurement was performed through an automated colorimetric assay based on the Griess reaction. The Griess reagent was prepared using solutions A (0.1% N-(1- napthyl) ethylenediamine dihydrochloride in H_2O) and B (1% sulfanilamide in 5% H_3PO_4). The mixture was then left at room temperature for 10 minutes, Griess reagent was mixed with the same volume of reaction mixture. The reaction mixture was prepared by mixing 100 ㎕ of the SNAP solution with vitamin C (Vit. C) or bee venom. This mixture was kept in a humidified incubator containing 5% CO_2 and 95% O_2 at 37℃ for 1.5, 3, 6, and 12 hours. Then the nitrite concentration was determined by measuring the absorbance of the reaction mixture at 540 nm using a microplate reader following the addition of the Griess reagent. In

all test sets, the NO_2^- concentrations in wells containing SNAP only were used as a blank control.

7. Statistical Analysis

The results are expressed as mean ± S.E.M. Statistical analysis was performed using the Statistical Package for Social Sciences software SAS (version 6.1.2). Significance was evaluated by one-tailed Student's t test between the control and test groups and Duncan's multiple range test between the test groups. Significance considered to be present at the P values specified in the figure legends.

Ⅲ. RESULTS

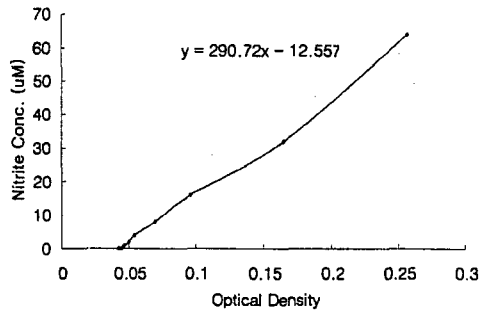
1. Standard Curve for NO_2^- Concentration

Standard solutions of various concentrations (0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 uM) were made using sodium nitrite. Using these the following formula was derived:

$$y = 290.72x - 12.557$$

The standard curve for nitrite concentration is showed in Fig. 1 with respect to nitrite concentration.

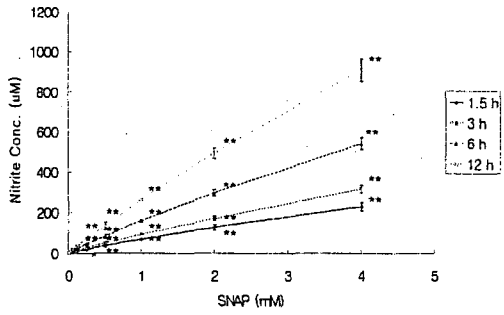
Fig. 1. Standard curve for nitrite concentration. The formula shown in the graph was derived and was subsequently used to estimate nitrite concentration from UV absorbance (540 nm).



2. NO_2^- Concentration Following Addition of SNAP Alone at Various Concentrations

Solutions containing SNAP at various concentrations (0.05, 0.125, 0.25, 0.5, 1, 2 and 4 mM) were incubated for 1.5, 3, 6 and 12 hours. The nitrite concentrations of the solutions are shown in Fig. 2. NO_2^- concentration was shown to increase in a time-and concentration-dependent manner.

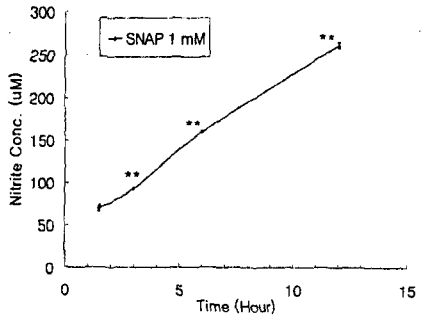
Fig. 2. Nitrite concentration 1.5, 3, 6 and 12 hours after treatment with SNAP at 0.05, 0.125, 0.5, 1, 2 and 4 mM. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. *p<0.05; **p<0.01 compared to results of corresponding SNAP treatment at 0.05 mM.



3. NO_2^- Concentration At Various Time Points Following Addition of 1 mM of SNAP

A solution containing 1 mM of SNAP was incubated for 1.5, 3, 6 and 12 hours. After incubation nitrite concentration was obtained by measuring the absorbance at 540 nm using a microplate reader. The results are shown in Fig. 3. NO_2^- concentration was shown to increase in a time-dependent manner.

Fig. 3. Linear plot showing variation of nitrite concentration following treatment with 1mM SNAP with respect to time. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. ** p<0.01 compared to the results obtained 1.5 hours after treatment with 1 mM SNAP.

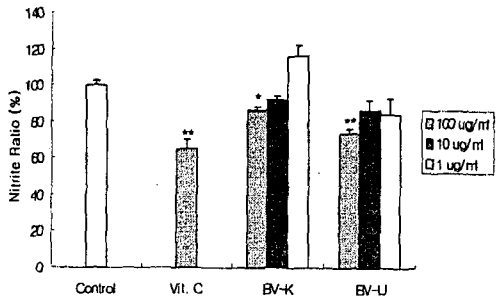


4. NO Scavenging Effect of Bee Venom

1) Nitrite Ratios 1.5 Hours After Addition of SNAP and Agent

Each agent was added to a solution containing SNAP, and the mixture was incubated for 1.5 hours. The nitrite concentration as a percentage of the control concentration in the solution containing 125 mM Vit. C was 65.7±6.0%. The concentrations of nitrite in the solutions containing 1, 10, and 100 ㎍/㎖ of BV-K were 116.2±6.0, 92.0±2.6, and 86.7±1.5%, respectively, whereas the figures for 1, 10, and 100 ㎍/㎖ of BV-U were 84.3±8.6, 86.2±6.2, and 74.0±2.2%, respectively(Fig. 4).

Fig. 4. Nitrite ratios 1.5 hours after the application of 1 mM SNAP and the respective agent. BV-K (whole bee venom of Korean bees) and BV-U (whole bee venom from USA) were given at concentrations of 1, 10 and 100 ㎍/㎖. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. Vit. C : 125 mM. *p<0.05, **p<0.01 compared to the control group.

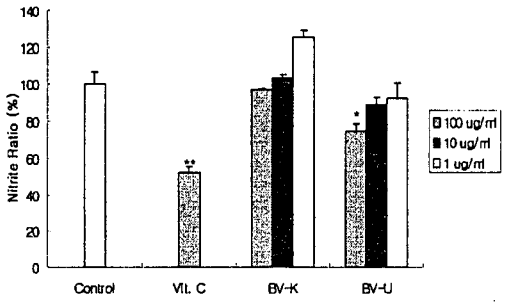


2) Nitrite Ratios 3 Hours After Addition of SNAP and Agent

Each agent was added to a solution containing SNAP, and the mixture was incubated for 3 hours. The nitrite concentration as a percentage of the control concentration in the solution containing 125 mM Vit. C was 51.8±9.3%. The concentrations of nitrite in the solutions containing 1, 10, and 100 ㎍/㎖ of BV-K were 125.5±4.1, 97.3±0.4, and 103.8±1.9%, respectively, whereas the figures for 1, 10, and 100 ㎍/㎖ of BV-U were 78.8±13.1, 79.6±16.7, and 66.5±10.9%, resp-

ectively (Fig. 5).

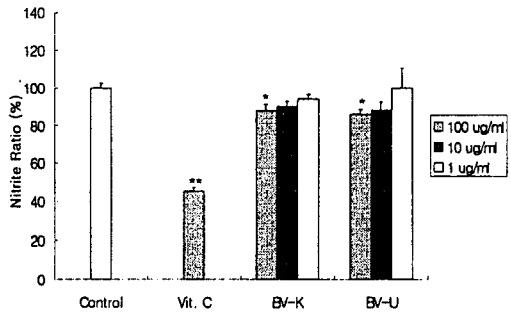
Fig. 5. Nitrite ratios 3 hours after application of and the 1 mM SNAP and the respective agent. BV-K (whole bee venom of Korean bees) and BV-U (whole bee venom from USA) at concentrations of 1, 10 and 100 ㎍/㎖. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. Vit. C : 125 mM. **p<0.01 compared to the control group.



3) Nitrite Ratios 6 Hours After Addition of SNAP and Agent

Each agent was added to a solution co-ntaining SNAP, and the mixture was incubated for 6 hours. The nitrite concentration as a percentage of the control concentration in the solution containing 125 mM Vit. C was 45.3± 1.5%. The concentrations of nitrite in the solutions containing 1, 10, and 100 ㎍/㎖ of BV-K were 94.3±2.4, 90.4±2.8, and 88.3± 2.9%, respectively, whereas the figures for 1, 10, and 100 ㎍/㎖ of BV-U were 100.1±10.4, 88.4±4.3, and 86.1±2.8%, respectively (Fig. 6).

Fig. 6. Nitrite ratios 6 hours after application of and the 1 mM SNAP and the respective agent. BV-K (whole bee venom of Korean bees) and BV-U (whole bee venom from USA) were given at concentrations of 1, 10 and 100 ㎍/㎖. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. Vit. C : 125 mM. **p<0.01 compared to the control group.

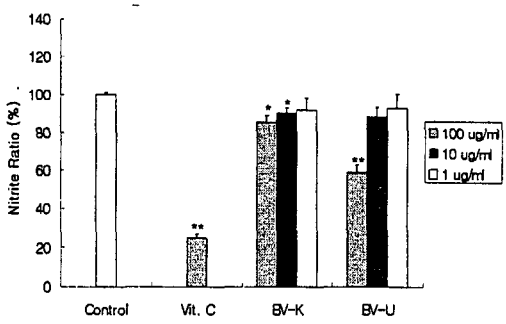


4) Nitrite Ratios 12 Hours After Addition of SNAP and Agent

Each agent was added to a solution con-taining SNAP, and the mixture was incubated for 6 hours. The nitrite concentration as a percentage of the control concentration in the solution containing 125 mM Vit. C was 24.8± 2.3%. The concentrations of nitrite in the solutions containing 1, 10, and 100 ㎍/㎖ of BV-K were 91.8±6.2, 90.1±3.0, and 85.6± 3.2%, respectively, whereas the figures for 1, 10, and 100 ㎍/㎖ of BV-U were 92.8±7.5, 88.3±5.1, and 59.1±3.8%, respectively (Fig. 7).

Fig. 7. Nitrite ratios 12 hours after application of and the 1 mM SNAP and the respective agent. BV-K (whole bee venom of Korean bees) and BV-U (whole bee venom from USA) were given at concentrations of 1, 10 and 100 ㎍/㎖. Each data point is represented as mean (±SEM). (n=3). Data were obtained from at least 3 independent

repetitions. Differences of significance as calculated via Student's t-test are marked with asterisks. Vit. C : 125 mM. *p<0.05, **p<0.01 compared to the control group.



IV. DISCUSSION AND CONCLUSIONS

NO acts as a mediator in neurotransmission, long term potentiation and depression, brain development[9], and various other processes in the cardiovascular, immune and nervous systems[10,11]. NO is synthesized by a family of enzymes that are collectively called nitric oxide synthase (NOS) (EC 1.14.13.49). Three isoforms (nNOS, iNOS and eNOS) have been identified; these enzymes were found to be heme-containing flavoproteins employing L-arginine as a substrate and requiring NADPH, flavin adenine dinucleotide and tetrahydrobiopterin as cofactors[12,13]. NO plays an important role in neuronal cell death during cerebral ischemia[14], Alzheimer's disease[15], Huntington's disease[16], and Parkinson's disease[17]. It has been demonstrated that cortical NO levels increase severalfold after middle cerebral arterial occlusion and that a significantly higher level of NOS activity persists over an extended period[18,19]. The initial burst of NO generation is apparently mediated by the constitutively expressed neuronal nitric oxide synthase (nNOS)[18,20]. This calcium-dependent isoform of NOS is stimulated in response to N-methyl-D-aspartate (NMDA) receptor-ion channel activation[21]. Subsequent and sustained NO production during ischemic injury is apparently attributed to an increased expression of nNOS[22] and the induction of the inducible NOS gene[23]. NO and its oxidative metabolites, e.g., peroxinitrite, have been implicated in the initiation and promotion of neuronal cell death and ischemic brain injury[24]. Parkinson's disease is characterized by a loss of dopaminergic neurons in the mesencephalon. Although the mechanism of this neuronal loss is still unknown, oxidative stress is very likely involved in the cascade of events leading to neuronal cell death[17]. NO produces an increase in intracellular cyclic guanosine 3',5'- monophosphate (cGMP) through activation of soluble guanylate cyclase. In 1977, NO was first shown to stimulate the soluble guanylate cyclase in homogenates of animals' brain[25,26].

NO is deeply involved in nociception in the central nervous system, at both the spinal and supraspinal levels. Intrathecal injection of L-arginine and NO-releasing compounds produced hyperalgesia during tail flick tests and in the formalin pain model[27,28]. This finding is supported by the demonstration of increased NOS-like immunoreactivity in lumbar dorsal root ganglion neurons of several animal models of neuropathic pain[29]. However, hyperalgesia

produced by chronic ligature of the sciatic nerve in rats is accompanied by a reduction in both NOS activity in central terminals of primary afferents[30] and neuronal NOS- positive cells in the dorsal horn[31].

In the present study we used SNAP, an NO donor, to examine the effects of exogenous NO. We found that treatment with Vit. C and bee venom reduce NO. The NO scavenging effect was measured through an automated colorimetric assay based on the Griess reaction. The concentration of NO_2^- resulting from the addition of SNAP only increased significantly in a dose- and time-dependent manner. When 125 mM Vit. C was added the concentration of nitrite as a percentage of the control value was 65.7±6.0, 51.8±9.3, 45.3±1.5 and 24.8±2.3% after 1.5, 3, 6, and 12 hours. This result shows that Vit. C is powerful NO scavenger, and this is consistent with previous reports[32,33]. With BV-K and BV-U significant NO scavenging effects were observed as well, with the concentration of 100 ㎍/㎖, rather than those of 10 ㎍/㎖ and 1 ㎍/㎖, showing the greatest effect. After 12 hours a greater level of scavenging effect was still present with BV-K than was with BV-U.

These results imply that bee venom may have a bearing on NO-induced pain, inflammation, edema, arthritis, and immune responses and that scavenging of the NO which has diffused into the synaptic cleft from neurons might postpone the initiation of hyperalgesia. It is also suggested that bee venom have the preventive effect against NO toxicity and could protect from cell death due to oxidant stress and facilitate the recovery of cellular viability. Further long-term studies are needed to elucidate the precise molecular mechanism of bee venom for acua-acupuncture on free radical damage.

Acknowledgments

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V. REFERENCES

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