원 저

Scavenging Effect of *Hominis Placenta* Herbal Acupuncture Solution on Nitric Oxide

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국문초록

자하거 약침액의 Nitric Oxide에 대한 소거 효과

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목적 : 본 연구는 최근 임상에서 많이 사용하는 자하거 약침액의 nitric oxide(NO)에 대한 소거 효과를 분석하기 위하여 실행되었다.

방법 : 양성대조군으로 비타민C와 실험군으로 자하거 약침액에 NO를 분비하는 S-nitroso-N-acetylpenicillamine(SNAP)을 투여한 후 NO의 농도를 540nm 파장의 자외선 흡수량을 측정하여 평가하였다.

결과 : 실험에 사용된 자하거 약침액의 NO 소거 효과는 강력한 항산화제인 비타민C보다 우수하지는 못하였으나 0.005 및 0.001mg/ml 농도에서 12시간 동안 SNAP를 투여한 후 생성된 NO를 유의성 있게 소거하는 효과가 나타났다.

결론 : 이상의 결과를 통하여 자하거 약침액이 NO를 소거하는 효과가 있는 것이 확인이 되었으나 추후 농도별 실험과 다른 시료와의 비교실험이 더 요구된다.

핵심단어 : Hominis Placenta, herbal acupuncture solution, nitric oxide(NO). S-nitroso-N-acetylpenicillamine (SNAP)

· This study was supported by Korea Institute of Oriental Medicine and grant number(R12-2003-002-03003-0) from the bagic research program of the Korea Science and Engineering Foundation
· 접수 : 2007년 7월 13일 · 수정 : 2007년 7월 24일 · 채택 : 2007년 7월 25일
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Ⅰ. Introduction

Free radicals from exogenous or endogenous sources damage tissue components. Since tissue damage by free radical increases with age, the reactive oxygen species(ROS) such as hydrogen peroxide (H_2O_2), nitric oxide(NO) and superoxides have been implicated in the pathogenesis of aging-related diseases such as cardiovascular disease, cancer, arthritis and neurodegenerative diseases[1,2]. In the central nervous system NO may play important roles in neurotransmitter release, neurotransmitter reuptake, neurodevelopment, synaptic plasticity, and regulation of gene expression. However, excessive production of NO following a pathologic result can lead to neurotoxicity. That neurotoxicity can make a variety of neurologic disorders, including stroke, Parkinson's Disease and dementia[3].

Herbal acupuncture therapy has been used many years ago in traditional oriental medicine. First record on herbal acupuncture treatment dated back to 168 B.C.[4]. People have been using herbal chemical stimulation on the body points for many centuries, but injection of the extract dates back to 1960's. Herbal acupuncture therapy is a new type of acupuncture treatment method that incorporates acupuncture and herbal medicine to stimulate the acupuncture point. In other words herbs are processed and extracted to make injectable fluid and small amount of this fluid is injected into the acupuncture point believed to be effective to achieve both efficacy of acupuncture and herbal medicine. Hominis Placenta herbal acupuncture is one of the methods of herbal acupuncture and this therapy is being used to relieve pain and to cure inflammatory disease.

The chemistry and pharmacology of herbal acupuncture is not verified yet. and now the effectness of herbal acupuncture on rheumatoid arthritis and dementia has been reported[5,6]. However, few reports have been focused about the anti-oxidant effects of herbal acupuncture. Moreover, its role is not clearly defined yet. Thus, we examined the effects of Hominis Placenta herbal acupuncture an attempt to elucidate the possible underlying mechanism of its action. The purpose of this present study was to investigate whether Hominis Placenta herbal acupuncture scavenge NO or not.

Ⅱ. Materials and methods

1. Herbal Acupuncture Solution Preparation

Hominis Placenta extract was prepared as previous methods[7] in Korean institute of herbal acupuncture. 200kg of healthy placental chorionic parenchyma was obtained from full-term births, was rinsed and the tissue fat was removed using acetone. And it was vacuum dried to obtain about 14kg of skimmed particulate chorionic tissue. It was heated after pepsin, hydrochloric acid and purified water was added and the pH readjusted to 1.8 using hydrochloric acid. The liquid phase was extracted and the supernatant was adjusted with 80 l of purified water, and the solution was sterilized by heat.

Activated carbon was added, then it was stirred and filtered. Ion exchange resin was added to the filtrate to reach pH 5 and it was filtered again. After the volume adjusted to 100 l with purified water, it was filtered using a Millipore® filter. The filtrate was poured into washed, sterilized and dried vials and then the liquid was capped and sterilized by 121°C for 20 minutes in an autoclave. From above step final concentration of Hominis Placenta extract was 0.01 mg/ml.

2. Chemicals and Apparatus

S-nitroso-*N*-acetylpenicillamine(SNAP) was purchased from Sigma(USA). Sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride for 50mM phosphate buffer saline, N-(1-Napthyl) Ethylenediamine dihydrochloride, Sulfani-

lamide and H_3PO_4 for Griess reagent were also purchased from Sigma. Ultra violet (UV) absorbance was estimated at 540nm with ELISA microplate reader(Molecular Device, USA).

3. Standard Curve of $NO2^-$ Concentration by Sodium Nitrite

Firstly, 0.031, 0.063, 0.125 and 0.25uM of standard solution was made with sodium nitrite ($NaNO_2$). And the standard curve was induced by using the known concentration of sodium nitrite. NO_2^- concentration was obtained by measuring the absorbance at 540nm in a microplate reader (data not shown). Then NO_2^- concentration was determined by calculating with the formular, y = 290.72x - 12.557.

4. SNAP Preparation

SNAP was prepared as previously reported[8]. SNAP was prepared by mixing 10mM N-acetyl-penicillamine in methanol-1 N HCl(20ml each) and 20mM $NaNO_2$ in distilled water (20mM) during 20minutes with vigorous stirring at 25℃. After 15minutes more well was washed with distilled water and air-dried to give deep green crystals.

5. NO_2^- Concentration by SNAP Alone

To determine NO_2^- concentration by SNAP alone 1 mM of SNAP was incubated in a humidified incubator containing at 37℃ 5% CO_2 and 95% O_2 for 1.5, 3, 6 and 12hours. After incubation each solution was added with Griess reagent. Nitrite concentration was obtained by measuring the absorbance at 540 nm in a microplate reader.

6. Measurement for NO Scavenging Effect

Nitrite measurement was performed by using an automated colorimetric assay based on the Griess reaction after treatment with 1 mM SNAP and vitamin C(Vit. C) or SNAP and *Hominis Placenta* herbal acupuncture solution. Griess reagent was prepared with solution A0.1% N-(1-Napthyl) Ethylenediamine dihydrochloride in H_2O and solution B(1% sulfanilamide in 5% H_3PO_4). It was reacted at room temperature for 10minutes, Griess reagent was mixed with the same volume of reaction mixture. The reaction mixture was prepared by mixed 100㎕ of SNAP with Vit. C or SNAP with *Hominis Placenta* herbal acupuncture solution. And this mixture were kept in a humidified incubator containing at 37℃ 5% CO2 and 95% O2 for 1.5, 3, 6, and 12hours. Then nitrite concentration was determined by measuring the absorbance of remained nitrite in media at 540nm in a microplate reader. In all experiments, NO_2^- concentration in wells containing medium only was also measured as a blank control.

7. Statistical Analysis

The results are expressed as the mean ± S.E.M. Statistical analysis was performed by using Statistical Package for Social Science software SAS program(version 8). Significance level was evaluated by one-tailed Student's t test between control and test group. Significance was tested at P values specified in the figure legends.

Ⅲ. Results

1. 1mM of SNAP According to Time

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

2. NO Scavenging Effect at 12Hours

Samples were treated for 12 hours with SNAP and *Hominis Placenta* herbal acupuncture solution. Percentage control of nitrite of 125mM Vit. C was

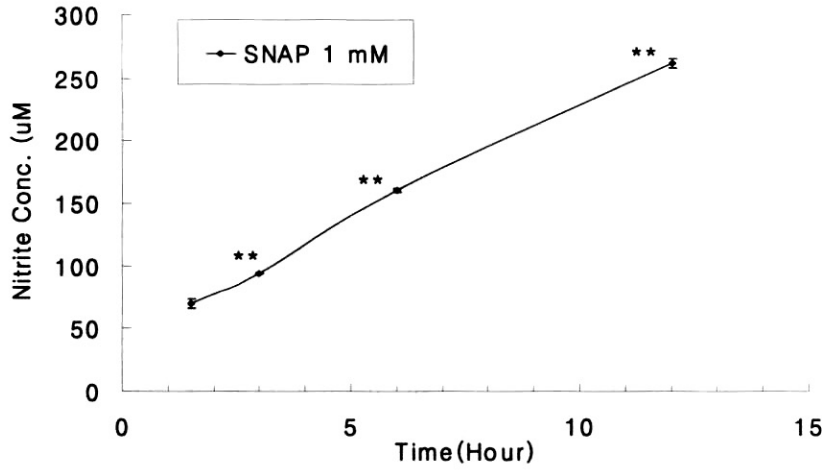

Fig. 1. Linear graph for variation of nitrite concentration after SNAP 1mM treatment according to time

Each point represents the mean(±SEM). These data were obtained from at least 3 time repetition. Significant differences calculated via Student's t-test are marked with asterisks. **p<0.01 vs. SNAP 1mM treatment at 1.5hours.

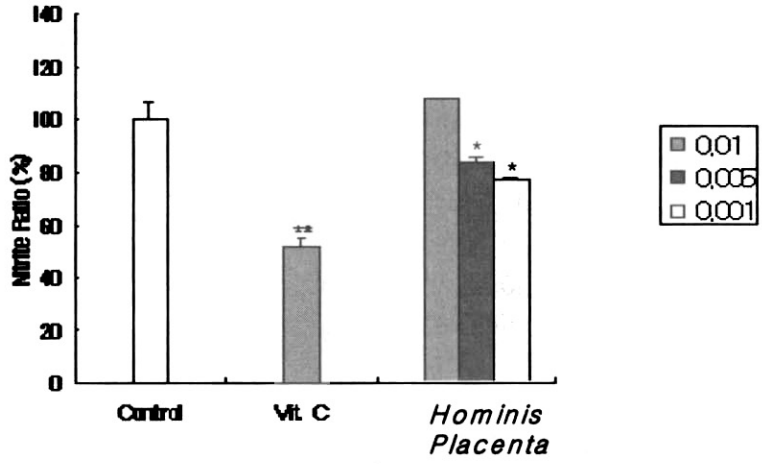

Fig. 2. Nitrite ratio at 12hours after 1mM SNAP and drug treatment

Hominis Placenta herbal acupuncture solution were treated at 0.01, 0.005 and 0.001㎎/㎖ of concentration. Each bar represents the mean (±SEM). These data were obtained from at least 3time repetition. Significant differences calculated via Student's t-test are marked with asterisks. *p<0.05; **p<0.01 vs. control group.

53.8±4.3% in comparison with 100±8.9% of control. Percentage control of nitrite of 0.01, 0.005 and 0.001 ㎎/㎖ of Hominis Placenta herbal acupuncture solution was 106.5±1.8, 84.3±4.1 and 79.1±2.6% respectively(Fig. 2). There was significant difference in concentration of 0.005 and 0.001㎎/㎖ of Hominis Placenta herbal acupuncture solution in comparison with control($p<0.05$).

Ⅳ. Discussion

NO is deeply involved in the nociceptive processing in the central nervous system, at both spinal and supraspinal levels. Intrathecal injection of *L*-arginine and NO-releasing compounds produced hyperalgesia in tail flick test and formalin pain model[9,10]. NO seems to play a role in the induction of nociception also because NOS-like immunoreactivity in

lumbar dorsal root ganglion neurons is increased in several animal models of neuropathic pain[11]. However, the hyperalgesia produced by chronic ligature of the sciatic nerve in rats is accompanied by reduction of both NOS activity in central terminals of primary afferents[12] and neuronal NOS-positive cells in the dorsal horn[13].

In addition NO acts as a mediator that plays a role in neurotransmission, long term potentiation, depression, brain development[14] and in the cardiovascular, immune and nervous systems[15,16]. NO is synthesized by a family of enzymes that are collectively called NOS (EC 1.14.13.49). Three isoforms (nNOS, iNOS and eNOS) of NOS 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[17,18]. NO plays an important role in neuronal cell death during cerebral ischemia[19], Alzheimer's disease[20], Huntington's disease[21] and Parkinson's disease[22]. Especially in cerebral ischemia, it has been demonstrated that cortical NO levels increase severalfold after middle cerebral artery occlusion and a significantly higher level of nitric-oxide synthase(NOS) activity is sustained over an extended period[23,24]. The initial burst of NO generation is apparently mediated by the constitutively expressed neuronal nitric-oxide synthase(nNOS)[23,25]. This calcium-dependent isoform of NOS is stimulated in response to N-methylD-aspartate(NMDA) receptor ion channel activation[26]. Subsequent and sustained NO production during ischemic injury is apparently attributed to an increased expression of nNOS[27] and induction of inducible NOS gene[28]. NO and its oxidative metabolites, e.g., peroxinitrite have been implicated in the initiation and promotion of neuronal cell death and ischemic brain injury[29]. Although the mechanism of the neuronal loss is still unknown, oxidative stress is very likely involved in the cascade of events leading to neuronal cell death[22]. NO is a free radical which is synthesized from *L*-arginine and molecular oxygen by NO synthase. It 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[30,31].

In the present study we used SNAP, a NO donor, to examine the effects of exogenous NO. We found that treatment with Vit. C and Hominis Placenta herbal acupuncture solution scavenge NO. NO scavenging effect was measured by using an automated colorimetric assay based on the Griess reaction after treatment with SNAP and Vit. C or SNAP and Hominis Placenta herbal acupuncture solution for 1.5, 3, 6 and 12 hours. NO_2^- concentration resulted from SNAP significantly increased in dose and time-dependent manner(Fig. 1). In case of 125mM Vit. C percentage control of nitrite was 53.8±4.3% in comparison with 100±8.9% of control. This results mean that Vit. C plays a role as a powerful NO scavenger and is consentient to previous report[32,33]. In case of test group percentage control of nitrite of 0.01, 0.005 and 0.001㎎/㎖ of Hominis Placenta herbal acupuncture solution was 106.5±1.8, 84.3±4.1 and 79.1±2.6% respectively(Fig. 2). There was significant difference in concentration of 0.005 and 0.001 ㎎/㎖ of Hominis Placenta herbal acupuncture solution in comparison with control($p< 0.05$).

These results imply that *Hominis Placenta* herbal acupuncture may relieve NO-induced pain, inflammation, edema, arthritis and immune response in clicnic and scavenging of NO which has diffused into the synaptic cleft from neuron cells might attenuate the initiation of hyperalgesia. During neuropathological conditions, the production of NO can be either protective or toxic on the stage of the disease, the isoforms of NOS involved and the initial pathological event[21]. So Further long-term studies are needed about in which condition NO can be protective factor or toxic trigger. These studies suggest that *Hominis Placenta* herbal acupuncture may the preventive effect against NO toxicity and could protect from cell death due to oxidant stress and facilitate the recovery of cellular viability. But it needs for more evaluation on the

NO scavenging effect of Hominis Placenta herbal acupuncture solution and further long-term studies to elucidate the precise mechanism on free radical damage.

V. References

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