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진범 약침이 collagenase로 유도된 흰쥐 골관절염 모델에서 NOS 발현에 미치는 영향

김현수[1] · 서일복[2] · 박세근[3] · 김정선[3] · 서정철[4] · 최선미[5] · 구성태[5] · 김이화[1]

[1]세명대학교 한의과대학 경혈학교실, [2]해부학교실, [3]한방식품영양학과
[4]대구한의대학교 한의과대학 침구학교실
[5]한국한의학연구원 의료연구부

Effects of Aconitum pseudo-laeve on Nitric Oxide Synthase in the Periaqueductal Gray of Collagenase-induced Rat Osteoarthritis Model

Hyun-Soo Kim[1], Il-Bok Seo[2], Se-Keun Park[3], Jeongseon Kim[3], Jung-Chul Seo[4], Sun-Mi Choi[5], Sung-Tae Koo[5], Ee-Hwa Kim[1]

[1]*Dept. of Meridian & Acupoint,* [2]*Dept. of Anatomy,*
[3]*Dept. of Food & Nutrition in Oriental Medicine, College of Oriental Medicine, Semyung University ;*
[4]*Dept. of Acupuncture & Moxibustion, College of Oriental Medicine, Daeguhani University ;*
[5]*Department of Medical Research, Korean Institute of Oriental Medicine*

Abstract

목적 : 본 연구는 collagenase로 유도된 흰쥐의 골관절염 모델에서 진범약침자극이 흰쥐 dorsolateral periaqueductal gray (DL-PAG)에서 NOS 및 nNOS 발현에 미치는 영향을 관찰하였다.

방법 : 흰쥐의 관절강내로 collagenase 용액을 주사하여 골관절염 모델을 만들고 정상군, 대조군 및 진범약침군으로 실험군을 분류한 후, nNOS(neuronal NOS)와 NOS에 대하여 미치는 영향을 nNOS immunohistochemistry와 nicotinamide adenine dinucleotide phosphate-diaphorase(NADPH-d) 검사법을 통하여 조사하였다.

결과 : 골관절염이 유발된 흰쥐의 DL-PAG 영역에서 nNOS와 NOS의 발현억제가 관찰되었으며, 진범약침군이 collagenase로 유도된 골관절염에서 감소된 nNOS와 NOS의 발현이 증가되었다.

결론 : 본 연구를 통하여 진범약침자극은 골관절염이 유발된 흰쥐의 DL-PAG에서의 nNOS와 NOS의 발현에 영향을 미친다는 결과를 얻을 수 있었다.

Key words : Acupuncture, Aconitum pseudo-laeve, Collagenase, Nitric oxide Osteoarthritis, Periaquaductal gray

Ⅰ. Introduction

A common feature of chronic articular joint diseases is the eventual and irreversible tissue destruction associated with a permanent loss of joint mobility. Osteoarthritis is a painful degenerative joint disease and a major cause of disability and represents the most common disease in the aging population[1,2]. In addition, the osteoarthritis can originate from various

• 교신저자 : 김이화, 충북 제천시 신월동 산21, 세명대학교 한의과대학 경혈학교실, Tel. 043-649-1348, Fax. 043-649-1702, E-mail : kimeh@semyung.ac.kr
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causes, with trauma and concomitant synovitis of knee joint, and inflammatory mediator including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), matrix metalloproteinases (MMPs), and nitric oxide (NO) play important roles in the pathogenesis of osteoarthritis[3].

NO, synthesized from L-arginine through calcium-dependent pathways by nitric oxide synthase (NOS), is a free radical with signaling functions in the central nervous system (CNS). NO has been implicated in the regulation of autonomic functions, and it has been shown to play important roles in the neural, vascular, and immune systems (Dawson et al., 1992). Several isoforms of NOS exist and fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Of these, nNOS is mainly expressed in the CNS and has been implicated in signal transmission[4,5].

The midbrain periaqueductal gray (PAG) is a key component of the brain stem endogenous pain control circuit. At the PAG, the exogenous opiates and endogenous opioids induce antinociception by activating the so-called 'descending pain-control system', which inhibits transmission of pain signals at the spinal dorsal horn. The descending pain control system is activated by electrical stimulation and local injection of morphine-like narcotics or opioid peptides at the PAG[6-8]. In immunohistochemical studies, the presence of NOS-staining neurons has been demonstrated in the dorsolateral PAG (DL-PAG).

Aconitum pseudo-laeve has been widely used for the treatment of various diseases in Oriental medicine. Aconitum genus is mainly characterized by the presence of diterpene alkaloids and for this reason its roots have long been used as Oriental medicine against gout, neuralgia, cardiac failure, and articular rheumatism[9,10].

In the present study, the effect of aqueous extract of Aconitum pseudo-laeve on the expression of NOS and nNOS in the DL-PAG was investigated via nNOS immunohistochemistry, and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, which takes advantage of the fact that NADPH-d-positive neurons are the same as those containing NOS[4].

Ⅱ. Materials & Methods

1. Animals and Treatments

Male Sprague-Dawley rats weighing 200 ± 10 g (6 weeks of age) were used for the experiment. Each animal was housed at a controlled temperature (20 ± 2 ℃) and was maintained under light-dark cycles, each cycle consisting of 12 h of light and 12 h of darkness (lights on from 07:00 h to 19:00 h), with food and water made available ad libitum. The experimental procedures were performed in accordance with the animal care guidelines of the NIH and the Korean Academy of Medical Sciences. Animals were divided into three groups: the control group, the osteoarthritis (OA) group, the OA and Aconitum pseudo-laeve-treated group (n = 8 in each group).

To induce osteoarthritis in the experimental animals, a single intra-articular injection of collagenase (50 mg/kg in saline; Sigma Chemical Co., St. Louis, MO, USA) into the knee joint was given to each anesthetized animal on the first day and the third day of the experiment respectively, while animals of the control group received equivalent amounts of normal saline. Animals of the Aconitum pseudo-laeve-treated groups were administered per os (P.O.) with aqueous extract of Aconitum pseudo-laeve at the 5 mg/kg dose for 15 days.

2. Preparation of Aconitum pseudo-laeve

To obtain the water extract of Aconitum pseudo-laeve, 200 g of Aconitum pseudo-laeve was added to distilled water, and extraction was performed by heating at 80 ℃, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g (15 %), was dissolved in saline.

3. Tissue preparation

Animals were weighed and overdosed with Zoletil 50 (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). After a complete lack of response was observed, the rats were transcardially perfused with 50 mM phosphate-buffered saline (PBS) and then with 4 % paraformaldehyde in 100 mM phosphate buffer (PB) at pH 7.4. The brains were dissected, postfixed in the same fixative overnight, and transferred into a 30 % sucrose solution for cryoprotection. Serial coronal sections of 40 μ m thickness were made using a freezing microtome (Leica, Nussloch, Germany).

4. NADPH-d histochemistry

For NADPH-d activity, ten sections on average were selected from each brain in the region spanning from Bregma -5.30 mm to -8.30 mm according to the atlas by Paxinos and Watson[11]. In brief, free-floating sections were incubated at 37 ℃ for 60 min in 100 mM PB containing 0.3 % Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 0.1 mg/ml NADPH. The sections were then washed three times with PBS and mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount.

5. nNOS immunohistochemistry

For analyzing the level of nNOS expression, ten sections on average were selected from each brain in the region spanning from Bregma -5.30 mm to -8.30 mm according to the atlas by Paxinos and Watson[11]. Free-floating tissue sections were washed twice in 50 mM PBS and were then permeabilized in 0.2 % Triton X-100 for 30 min. After washing twice with PBS, sections were incubated overnight with mouse anti-nNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000. Sections were washed twice in PBS and incubated for 1 h with biotinylated anti-rabbit antibody (1:200). Bound secondary antibody was then amplified with Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The antibody-biotin- avidin-peroxidase

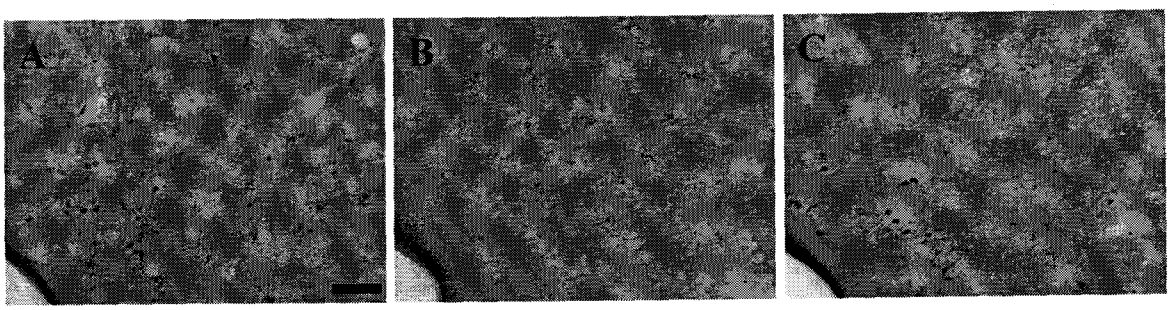

Fig. 1. Photomicrographs of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cells in the dorsolateral periaqueductal gray (DL-PAG). Sections were stained for NOS (blue). Scale bar represents 100 μ m. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Aconitum pseudo-laeve-treated group.

complexes were visualized using 0.05 % diaminobenzidine. The sections were mounted onto gelatinized glass slides and air-dried, and cover slides were mounted using Permount.

6. Data analysis

The area of PAG was measured using Image-ProPlus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA). The total numbers of nNOS-positive and NADPH-d-positive neurons in the PAG were counted hemilaterally under a light microscope (Olympus, Tokyo, Japan), and the results were expressed as numbers of nNOS-positive and NADPH-d-positive cells per section of the area of the PAG region.

7. Statistical analysis

Data were analyzed using SPSS (version 10.0) by one-way analysis of variance (ANOVA) followed by Student's t-test, and results were expressed as mean standard error mean (S.E.M.). Differences were considered significant for $p < 0.05$.

Ⅲ. Results

1. Effects of Aconitum pseudo-laeve on the number of NADPH-d-positive cells in the DL-PAG

NADPH-d-positive cells were mainly localized in the dorsolateral area of the PAG, consistent with the findings of Rodella et al.[12].

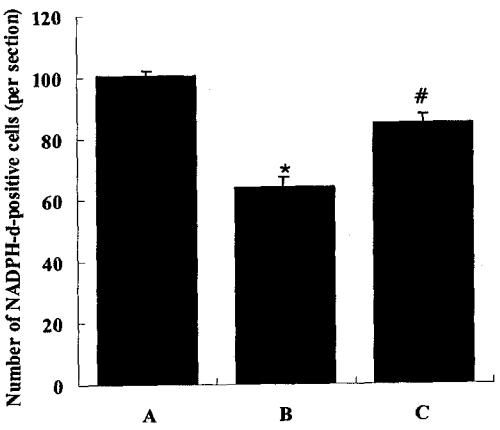

Fig. 2. Mean number of NADPH-d-positive cells in the DL-PAG in each group. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Aconitum pseudo-laeve-treated group. *represents $P < 0.05$ compared to the control group. #represents $P < 0.05$ compared to the Osteoarthritis group.

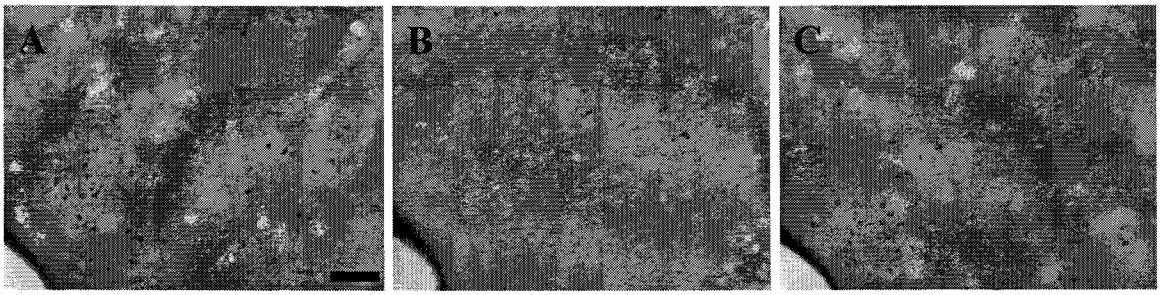

Fig. 3. Photomicrographs of neuronal nitric oxide synthase (nNOS)-positive cells in the dorsolateral peri-aqueductal gray (DL-PAG). Sections were stained for NOS (reddish brown). Scale bar represents 100 μ m. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Aconitum pseudo- laeve-treated group.

The number of NADPH-d-positive cells in the control group was 100.44 ± 1.56/section. This number was decreased to 63.96 ± 3.1/section in the OA group but was increased again to 84.57 ± 3.20/section in the OA and Aconitum pseudo-laeve-treated group.

2. Effects of Aconitum pseudo-laeve on the number of nNOS-positive cells in the DL-PAG

A similar pattern observed for NOS expression. The number of nNOS-positive cells in the control group was 109.00 ± 2.57/section. This number was decreased to 86.91 ± 1.75/section in the OA group but was increased again to 103.14 ± 2.13/section in the OA and Aconitum pseudo-laeve-treated group.

Ⅳ. Discussion

Herbs in the Aconitum family have mostly been used for increasing the health effects of traditional medicine in Korea. Recently, the cardioprotective effect Aconitum family may be in part related to scavenging of hydroxyl radicals or inhibition of lipid peroxidation[13] . Shi et al.[14] reported that aconitines, a total alkaloids isolated from the main root of Aconitum carmichaeli, have been found useful in inhibiting significantly immune inflammations, rat's

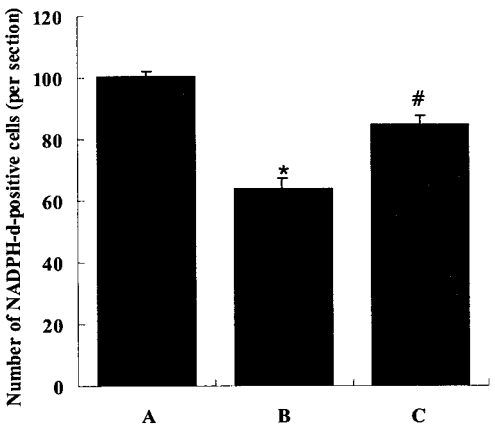

Fig. 4. Mean number of neuronal nitric oxide synthase (nNOS)-positive cells in the DL-PAG in each group. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Aconitum pseudo-laeve-treated group. *represents $P < 0.05$ compared to the control group. #represents $P < 0.05$ compared to the Osteoarthritis group.

delayed skin hypersensitivity and adjuvant arthritis.

Osteoarthritis is known to be induced through several complex mechanism such as progressive erosion of articular cartilage, proteoglycan degradation, and disruption of the collagen network, leading to a progressive destruction of joints and functional loss[15]. Kikuchi et al.[16] reported that intraarticularly injected collagenase digests cartilage directly and stimulates an inflammatory reaction in joint tissues at an early stage, after wich the cartilage degeneration proceeds.

NO has been implicated in the pathogenesis of osteoarthritis and rheumatoid arthritis in experimental animal models of arthritis[17,18]. Previous studies have shown that mechanical stress is an important modulator of NO in several cell types, including endothelial cells[19], osteocytes[20], and osteoblasts[21]. NO production is increased in chondrocytes exposed to fluid shear stress, while in isolated chodrocytes embedded in agarose, intermittent compression can decrease NO production[22]. Possible role of NO as an inflammatory mediator remains controversial. In particular, many studies have been made on NO release in osteoarthritis-affected chondrocytes or osteoblasts, however no report has been made on the effect of osteoarthritis on the activity of PAG containing NOS, yet. In the present results, decreased expression of nNOS and NOS was observed in the DL-PAG of collagenase-induced osteoarthritis rats, providing physiological evidence of the involvement of NOS in the osteoarthritis. Because of the involvement of neuropathogenesis in collagenase-induced osteoarthritis, it appears logical that drug which modulates NOS may be of use in reducing osteoarthritis-induced neuronal change.

In the present results, the aqueous extract of Aconitum pseudo-laeve treatment was enhanced the osteoarthritis-induced suppression in the expression of nNOS and NOS in the DL-PAG. The present results suggest that Aconitum pseudo-laeve can exert its analgesic effects probably by modulating NOS expressions, and it is very possible that Aconitum pseudo-laeve can offer a valuable mode of therapy for the treatment of osteoarthritis.

V. CONCLUSION

In the present study, we investigated that the effect of aqueous extract of Aconitum pseudo-laeve on the expressions of nNOS and NOS in the DL-PAG area of rats with collagenase-induced arthritis. Suppressed exprssion of nNOS and NOS was detected in the DL-PAG of rats with osteoarthritis, and Aconitum pseudo-laeve treatment increased the suppressed expression of nNOS and NOS in the DL-PAG of rats with osteoarthritis.

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