대한경락경혈학회지 Vol.22, No.4, pp.109~116, 2005
The Korean Journal of Meridian & Acupoint

# 위령선 약침이 콜라겐으로 유도된 골관절염 모델에서 흰쥐의 PAG 영역에서 NOS 발현에 미치는 영향

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# Effects of Clematis mandshurica Rupr. on Nitric Oxide Synthase in the Periaqueductal Gray of Collagenase-induced Rat Osteoarthritis Model

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## Abstract

**목적** : 골관절염은 진통을 수반하는 퇴행성 관절질환이며, 장애를 일으키는 주요한 원인이 된다. 또한 노인들에 있어서 골관절염은 매우 흔한 질환이라 할 수 있다. Nitric oxide(NO)는 Nitric Oxide Synthase(NOS)에 의하여 칼슘의존성통로를 통하여 L-arginine 으로부터 합성되어지며, NO는 중추신경계에 있어서 중요한 세포사이의 전달자이다.

**방법** : 본 연구에서는 위령선 으로부터 추출한 액이 콜라겐으로 유도된 관절염에 걸린 쥐의 dorsolateral periaqueductal gray(DL-PAG) 영역에서 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, 위령선이 콜라겐으로 유도된 골관절염에서 감소된 nNOS와 NOS의 발현이 증가되었다.

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

**Key words** : Acupuncture, *Clematis mandshurica*, Collagenase, Nitric oxide Osteoarthritis, Periaquaductal gray

## Ⅰ. Introduction

Clematis mandshurica Rupr. (Ranunculaceae) has been widely used for the treatment of various diseases in oriental medicine. Herbs of Clematis family have been used to treat rheumatic arthritis and other inflammatory conditions, such as laryngitis, skin and breast

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· 투고 : 2005/11/30 · 수정 : 2005/12/13 · 채택 : 2005/12/19

infections[1,2].

Osteoarthritis is a painful degenerative joint disease and a major cause of disability and represents the most common disease in the aging population[3,4]. In addition, the osteoarthritis can originate from various causes, with trauma and concomitant synovitis of knee joint, and inflammatory mediator including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), matrix metalloproteinases (MMPs), and nitric oxide (NO) play important roles in the pathogenesis of osteoarthritis[5].

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[6]. 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[6,7].

The midbrain periaqueductal gray (PAG) is believed to be an important component in the descending (endogenous) pain control system[8]. The descending pain control system is activated by electrical stimulation and local injection of morphine-like narcotics or opioid peptides at the PAG[9-11]. In immunohistochemical studies, the presence of NOS-staining neurons has been demonstrated in the dorsolateral PAG (DL-PAG).

In the present study, the effect of aqueous extract of Clematis mandshurica 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[7].

## 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 Clematis mandshurica-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 Clematis mandshurica-treated groups were administered per os (P.O.) with aqueous extract of Clematis mandshurica at the 5 mg/kg dose for 15 days.

## 2. Preparation of Clematis mandshurica

To obtain the water extract of Clematis mandshurica, 200 g of Clematis mandshurica 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 $\mu$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[12]. 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[12]. 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 complexes were visualized using 0.05 % diaminobenzidine. The sections were mounted onto gelatinized glass slides and air-dried, and cover slides were

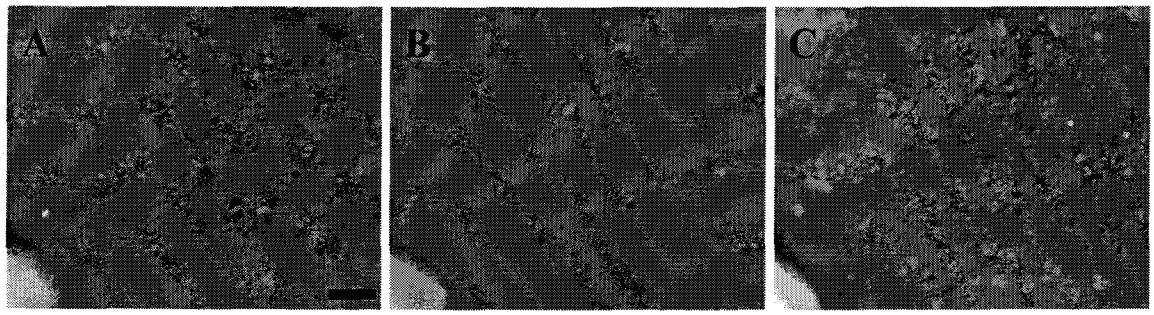


Fig. 1. Photomicrographs of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cells in the dorsolateral periaqueductal gray (DL-PAG). Sections were stained for NOS (blue). Scale bar represents 100 $\mu$ m. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Clematis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

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 Clematis mandshurica 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.[13].

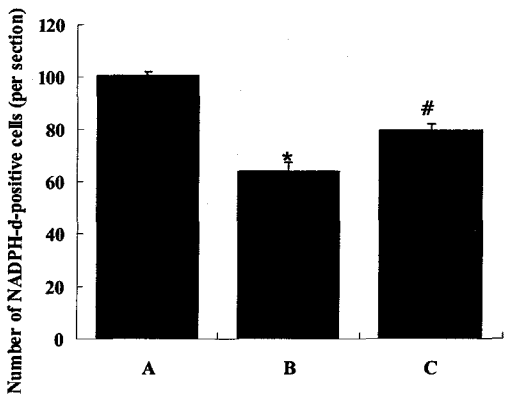


Fig. 2. Mean number of NADPH-d-positive cells in the DL-PAG in each group. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Clematis mandshurica-treated group. *represents $P < 0.05$ compared to the control group. #represents $P < 0.05$ compared to the Osteoarthritis group.

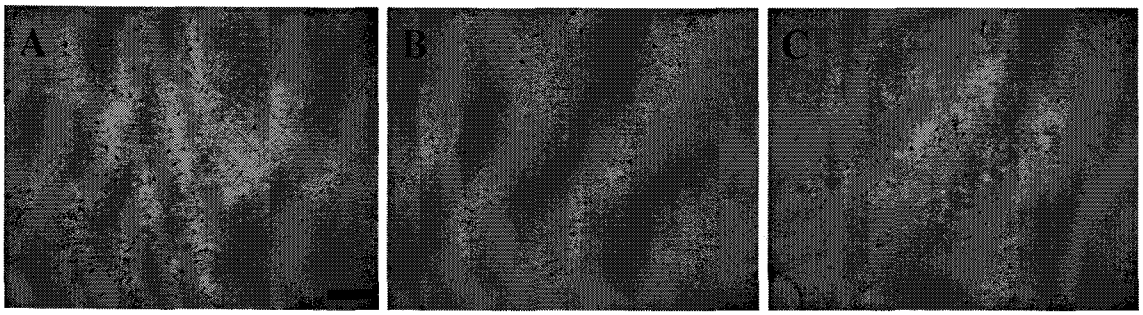


Fig. 3. Photomicrographs of neuronal nitric oxide synthase (nNOS)-positive cells in the dorsolateral periaqueductal gray (DL-PAG). Sections were stained for NOS (reddish brown). Scale bar represents 100 $\mu$ m. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Clematis mandshurica-treated 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 ± 3.10/section in the OA group but was increased again to 79.36 ± 2.57/section in the OA and Clematis mandshurica-treated group.

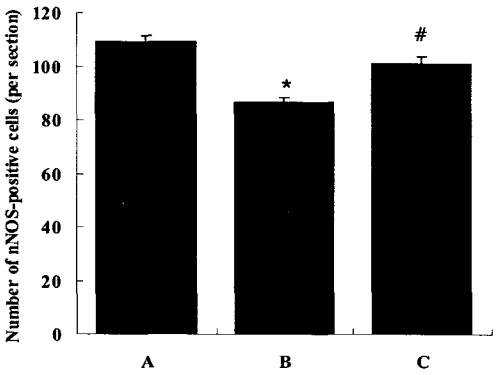


Fig. 4. Mean number of neuronal nitric oxide synthase (nNOS)-positive cells in the DL-PAG in each group. A; Control group, B; Osteoarthritis group, C; Osteoarthritis and Clematis mandshurica-treated group. *represents $P < 0.05$ compared to the control group. #represents $P < 0.05$ compared to the Osteoarthritis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

### 2. Effects of Clematis mandshurica on the number of nNOS-positive cells in the DL-PAG

A similar pattern was 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 101.06 ± 2.74/section in the OA and Clematis mandshurica-treated group.

## Ⅳ. Discussion

The purpose of the present study was to investigate whether aqueous extract of Clematis mandshurica administration alters NOS expression in the PAG region of collagenase-induced osteoarthritis model. In the present study, Clematis mandshurica modulated osteoarthritis-induced NOS expression associated with inflammation and pain in the PAG region of CNS.

Recently, alternative and complementary approaches such as the use of a wide array of

natural, herbal, nutritional, and physical manipulations are becoming popular. For instance, dietary supplements containing soy protein or Harpgophytum procumbens extract have been shown to have some efficacy against the symptoms of osteoarthritis[14,15]. Herbs of Clematis family possessed diuretic activity[16], antimicrobial activity[17], and anti-inflammatory activity[1,2]. In addition, purified extract from the mixture of three oriental herbs including Clematis mandshurica protect against collagenase-induced rabbit osteoarthritis models by reducing proteoglycan degradation in cartilage[18].

Osteoarthritis is the most common joint disorder and a major contributor to disability in the elderly[19]. Although there is a lack of agreement regarding the definition of osteoarthritis, it is generally viewed as a degenerative disorder involving cartilage degradation. Typically, the degenerative changes are accompanied by a local inflammatory component that accelerates the joint destruction.

NO has been implicated in the pathogenesis of osteoarthritis and rheumatoid arthritis in experimental animal models of arthritis[4,20]. Previous studies have shown that mechanical stress is an important modulator of NO in several cell types, including endothelial cells[21], osteocytes[22], and osteoblasts[23]. In articular cartilage, NO production is increased in chondrocytes exposed to fluid shear stress, while in isolated chodrocytes embedded in agarose, intermittent compression can decrease NO production[24]. Possible role of NO as an inflammatory mediator remains controversial. In particular, many studies have been made on NO release in osteoarthritis-affected chodrocytes or osteoblasts, however no report has been made on the effect of osteoarthritis on the activity of PAG containing NOS, yet. In the present, decreased expression of nNOS and NOS was observed in the DL-PAG of collagenase-induced osteoarthritis rats. 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.

## V. Conclusion

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

## Acknowledgements

This study is supported by Korea Ins-

titute of Oriental Medicine

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