Journal of the Korea Convergence Society
Vol. 10. No. 2, pp. 331-337, 2019

ISSN 2233-4890
https://doi.org/10.15207/JKCS.2019.10.2.331

Treatment with ultra-dilutions of *Arnica montana* increases COX-2 expression and PGE2 secretion in mouse chondrocytes

Yun Kyu Kim[1], Myeong Gu Yeo[2*]
[1]Researcher, Department of Medicine, University of Ulsan College of Medicine,
[2]Professor, Department of Integrative Medical Sciences, Nambu University

생쥐 연골세포에 *Arnica montana* 처리에 따른 COX-2 발현과 PGE2 분비 비교

김윤규[1], 여명구[2*]
[1]울산대학교 의과대학 전임연구원, [2]남부대학교 통합의료학과 교수

Abstract Objective: We studied the effects of 4x, 30x, 30c, and 200c homeopathic dilutions of *A. montana* on inflammation in primary cultured mouse chondrocytes.
Methods: Examined expression of Coll-2 and COX-2, and secretion of PGE2.
Results: Treatment with 4x, 30x, and 30c *A. montana* decreased mRNA expression of *Coll-2* and 30x *A. montana* increased mRNA expression of *COX-2*, while treatment with 30x and 30c *A. montana* increased protein expression of COX-2. Treatment with the 30c *A. montana* increased release of PGE2.
Conclusion: Treatment with *A. montana* induces dedifferentiation and inflammatory responses, including increased COX-2 expression and PGE2 secretion.

Key Words : Convergence, Arnica montana, Homeopathic medicine, COX-2, PGE2.

요 약 연구목적 : 4x, 30x, 30c, and 200c 농도의 동종약물 *Arnica montana* (*A. montana*)를 1차 배양된 생쥐 연골세포에 적용하여 염증관련 인자의 변화를 관찰하고자 하였다.
연구방법 : 본 연구는 collagen type II (Coll-2), cyclooxygenase-2 (COX-2) 발현 그리고 prostaglandin 2 (PGE2) 분비에 대해 조사하였다.
결과: 4x, 30x 그리고 30c의 *A. montana*를 처리하였을 때, *Coll-2*의 mRNA 발현이 감소하였으며, 30x *A. montana*의 경우 *COX-2* mRNA의 발현이 증가하였다. 또한 COX-2 단백질 발현은 30x와 30c의 *A. montana* 처리 시 증가함을 보였다. PGE2 분비 또한 30c에서 증가함을 관찰하였다.
결론 : *A. montana* 처리에 따라 생쥐의 연골세포의 분화 억제를 확인하였으며, 염증관련 인자인 COX-2 및 PGE2의 발현이 증가함을 확인하였다.

주제어 : 융합, Arnica montana, 동종의약물, COX-2, PGE2

*This study was supported by research funds from Nambu University (2016) and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07050192).
*Corresponding Author : Myeong Gu Yeo (mgy11@nambu.ac.kr)
Received January 15, 2019 Revised February 8, 2019
Accepted February 20, 2019 Published February 28, 2019

1. Introduction

Products derived from medicinal herbs are used to treat and prevent diseases. Various natural plant compounds are biologically active and thus of pharmacological importance[1]. The homeopathic medicine *A. montana* elicits anti- inflammatory effects and has been used to reduce pain relief in various inflammatory conditions such as sprains, bruises, gingivitis, and rheumatic complaints[2,3]. Homeopathic medicines are dilutions of active ingredients or are administered at reduced doses, and are prescribed according to the similia principle[4]. One of the main criticisms of homeopathic medicines is the lack of stringent quality control parameters, and symptomatic analogy must be identified. Many trials have investigated the effects of homeopathic medicines involving its mechanisms[5,6]. *A. montana* 6cH modulates acute inflammation in animal models[8]. Inflammation is a complex biological response of tissues to harmful stimuli, and its symptoms include pain, heat, redness, swelling, and loss of function. Although various homeopathic remedies elicit anti-inflammatory effects and there by alleviate pain and reduce swelling in animal models, the underlying molecular mechanisms are poorly understood.

Many studies have demonstrated the anti-inflammatory and immunomodulatory effects of homeopathic remedies using experimental animal models[7]. Homeopathic dilutions of *Rhus toxicodendron* elicit anti-inflammatory effects, which involve histamine and prostaglandin, and thereby improve arthritis. We previously reported that *Rhus toxicodendron* modulates anti- inflammatory mechanisms in primary cultured mouse chondrocyte[5].

Here, we investigated the effects of *A. montana* on inflammation in mouse chondrocytes. We performed reverse-transcriptase polymerase chain reaction (RT-PCR) to examine mRNA expression of *Coll-2*, which is a marker protein of differentiated chondrocytes. In addition, we examined mRNA and protein expression of COX-2, which is a major mediator of inflammation in arthritis. COX-2 is primarily involved in inflammation and is responsible for synthesis of prostanoids (prostaglandins and thromboxanes) involved in pathological processes [8-10]. COX-2 promotes release of the pro-inflammatory mediator PGE2, while COX-2 inhibitors suppress production of PGE2[11]. The present study demonstrates that treatment with *A. montana* increased mRNA and protein expression of COX-2 and secretion of PGE2 in primary cultured mouse chondrocytes, suggesting that this remedy modulates the inflammatory process.

2. Materials and methods

2.1 Preparation of reagents

Liquid dilutions of *A. montana* at 4x, 30x, 30c, and 200c were purchased from Boiron (Newtown Square, PA, USA) and used to 1:10 dilution according to the manufacturer's instructions. The liquid form of *A. montana* was supplied in 20% ethanol (EtOH). The cell culture medium was supplemented with EtOH alone at a final concentration of 0.5% (v/v) as a control. Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Primary culture of mouse chondrocytes

The mice were maintained under specific pathogen-free conditions, and experimental treatment was approved through Institute of Asan Life Science (Seoul, Korea, Animal Care and Use Committee). Articular chondrocytes were obtained from 8-day-old mice[12]. Cartilage was isolated from the femoral head, femoral condyle, and tibial plateau, and then digested with 0.2% (w/v) collagenase type II. Cells were grown in Dulbecco's modified Eagle's media (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.3 Cell proliferation and toxicity assays

Cell proliferation and toxicity were assessed using a Cell Titer 96 Non-radioactive Cell Proliferation Assay, which uses MTT (3-(4,5-di- methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and a Lactate Dehydrogenase (LDH) Assay Kit (Promega, Madison, WI, USA), respectively, according to the manufacturer's instructions. Briefly, cells were plated into 96-well plates at a density of 1.0×10^4 cells/wells and cultured for upto 24hr. Thereafter, cells were treated with 2% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr at 37°C in a humidified atmosphere containing 5% CO_2. Absorbance at 570 and 490 nm was measured in the MTT and LDH assays, respectively, using an ELISA reader (BioTek Instruments, Winooski, VT, USA). Data are the average results of three wells in one independent experiment, which was repeated fourtimes.

2.4 RT-PCR

Chondrocytes were grown in medium containing 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr. Total RNA was extracted using a RNeasy Kit (Qiagen, Austin, TX, USA) according to the manufacturer's instructions and reverse- transcribed into cDNA. Reverse transcription was performed using 1 μg total RNA and TOPscript RT DryMIX (Enzynomics, Seoul, Korea). PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) and Ampli*Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The following primers (Macrogen, Seoul, Korea) were used for RT-PCR: *glyceraldehyde phosphate dehydrogenase* (*GAPDH*, 587 bp), sense 5'-TCACGCCACCCAGAAGAC-3' and antisense 5'-TCACTGCCACCCAGAAGAC-3'; and *COX-2*, sense 5'-GGTCTGGTGCCTGGTCTGATGAT-3' and antisense 5'-GTCCTTTCAAGGAGAATGGTGC-3'. The PCR conditions were denaturation (95°C for 3 min), amplification and quantification (22 cycles of 95°C for 20 sec, 62°C for 10 sec, and 72°C for 30 sec to analyze *GAPDH*, and 28 cycles of 95°C for 20 sec, 63°C for 10 sec, and 72°C for 30 sec to analyze *COX-2*), followed by a final elongation (72°C for 5 min). The amplified PCR products were visualized by electrophoresis on 1.5% agarose gels. RT-PCR was performed in triplicate. Amplification of the target gene was normalized against that of *GAPDH* in the same reaction.

2.5 Immunoblot analysis

Chondrocytes were treated with 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr and lysed in lysis buffer (1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM pyrophosphate, and 2 mM Na_3VO_4) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) on ice for 10min. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by SDS-PAGE. Immunoblot analysis was performed using antibodies against COX-2 (R&D Systems, Minneapolis, MN, USA) and actin (Millipore, Billerica, MA, USA). Bound antibodies were detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL, USA) using a LAS 4000 mini biomolecular imager (GE Healthcare, Uppsala, Sweden).

2.6 PGE2 assay

Chondrocytes were stimulated with 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr. The level of PGE2 in the culture medium was assessed using a PGE2 Assay Kit (R&D Systems) according to the manufacturer's instructions. Data represent the average results of triplicates in one independent experiment, which was repeated four times.

2.7 Statistical analysis

Experiments were performed at least four times.

Values are presented as the mean ± standard error of the mean (SEM). Data were statistically analyzed using the independent samples t-test and an analysis of variance. $p<0.05$ was considered statistically significant.

3. Results

3.1 Effect of *A. montana* on the proliferation and viability of chondrocytes

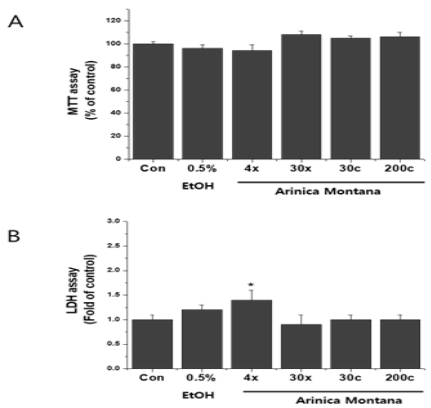

Fig. 1. Treatment with homeopathic dilutions of *A. montana* does not affect proliferation of chondrocytes or elicit cytotoxic effects. Cells were assessed by the MTT(A) and LDH(B) assays. Graphs show the mean ±SEM. *$p<0.05$. Con: untreated chondrocytes.

The effect of *A. montana* on proliferation of primary cultured mouse chondrocytes and its cytotoxic effects were evaluated using the MTT and LDH assays, respectively. Cells were analyzed following treatment with 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr. Treatment with homeopathic dilutions of *A. montana* did not affect the survival or proliferation of chondrocytes Fig. 1A and did not elicit cytotoxic effects, although 4x dilution showed cytotoxicity Fig. 1B.

3.2 Effect of *A. montana* on mRNA expression of *Coll-2* and *COX-2* in chondrocytes

Coll-2 and COX-2 function in joint inflammation in arthritis[13]. Cells were treated with 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr, and then mRNA expression of *COX-2* and *Coll-2* was examined by RT-PCR. Treatment with 4x, 30x, and 30c homeopathic dilutions of *A. montana* decreased mRNA expression of *Coll-2* Fig. 2, suggesting that it induced dedifferentiation of chondrocytes. Moreover, treatment with the 30x homeopathic dilution of *A. montana* significantly increased mRNA expression of *COX-2* Fig. 2A and Fig. 2C.

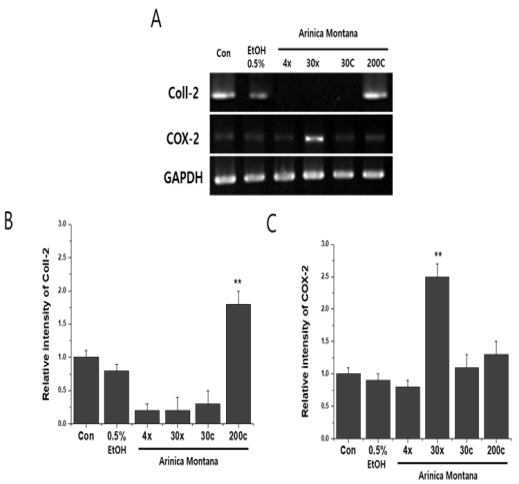

Fig. 2. Treatment with the 30x homeopathic dilution of *A. montana* increases mRNA expression of *COX-2* in chondrocytes. (A) cDNA was electrophoresed on an agarose gel. The band densities of *Coll-2*(B) and *COX-2*(C) were determined by densitometry. Target gene expression was normalized against that of *GAPDH*. Graphs show the mean ±SEM. ** $p<0.01$. Con: untreated chondrocytes.

3.3 Effects of *A. montana* on protein expression of COX-2 in chondrocytes

We next investigated the effects of *A. montana* on protein expression of COX-2 in chondrocytes. Cells were treated with 0.5% EtOH or the 4x, 30x, 30c, or 200c homeopathic dilution of *A. montana* for 48 hr, and then protein expression of COX-2 was examined by

immunoblotting. Treatment with 4x, 30x and 30c homeopathic dilutions of *A. montana* significantly increased protein expression of COX-2 in chondrocytes Fig. 3A and Fig. 3B. This result indicates that homeopathic dilutions of *A. montana* increase protein expression of COX-2 and thereby affect the inflammatory response.

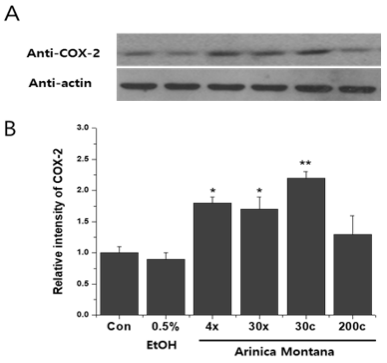

Fig. 3. Treatment with 4x, 30x, and 30c homeopathic dilutions of *A. montana* increases protein expression of COX-2 in chondrocytes. (A) Protein expression of COX-2 was analyzed by immunoblotting with an anti-COX-2 polyclonal goat antibody. The membrane was re-probed with an anti-actin antibody. (B) The band densities of COX-2 were determined by densitometry. The graph shows the mean ±SEM. * $p<0.05$, ** $p<0.01$. Con: untreated chondrocytes.

3.4 Effects of *A. montana* on PGE2 release by chondrocytes

Induction of COX-2 expression is closely associated with release of PGE2, and treatment with a COX-2 inhibitor reduces inflammation in animal models[9]. The level of PGE2 in the culture medium was examined following treatment of chondrocytes with homeopathic dilution of *A. montana* for 48 hr. Treatment with 30x and 30c homeopathic dilutions of *A. montana* increased the level of PGE2 in the culture medium Fig. 4, which was consistent with the effects on mRNA and protein expression of COX-2.

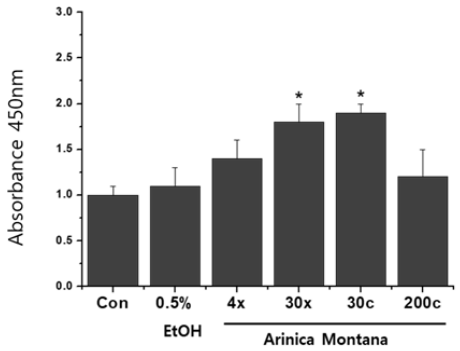

Fig. 4. Treatment with the 30c homeopathic dilution of *A. montana* increases release of PGE2 by chondrocytes. The level of PGE2 in the culture medium was measured. The graph shows the mean ±SEM. *$p<0.05$. Con: untreated chondrocytes.

4. Discussion

Many clinical trials and studies have demonstrated the effectiveness of homeopathic remedies using experimental animal models [14,15]. *A. montana*, which is one of the most common homeopathic remedies, has been used to alleviate joint pain. In its original, undiluted form, *A. montana* typically causes muscular, articular, and ligament pain and induces dermatitis[7]. However, the mechanisms underlying the effects of *A. montana* are unknown.

This study investigated the effects of *A. montana* on primary cultured mouse chondrocytes. To determine the effects of homeopathic dilutions of *A. montana* on inflammation, we measured mRNA expression of *COX-2* by RT-PCR. Treatment with the 30x homeopathic dilution of *A. montana* increased mRNA expression of *COX-2*, while treatment with 4x, 30x, and 30c homeopathic dilutions of *A. montana* decreased mRNA expression of *Coll-2*. COX-2 is dramatically upregulated during inflammation in patients with rheumatoid arthritis. A COX-2 inhibitor reduces inflammation[15, 16], and treatment with highly potent homeopathic remedies was reported to elicit a similar effect[15]. Although homeopathic dilutions of *A.*

montana also affected chondrocytes in the current study, the underlying mechanism is unclear.

Treatment with 30x and 30c homeopathic dilutions of *A. montana* increased PGE2 release. Previous studies suggested that a broad spectrum of inflammation mediators regulate COX-2 expression[8] and that PGE2, the major COX-2 product, is upregulated in various cell lines[17,18]. The results of the present study are consistent with previous findings demonstrating a correlation between COX-2 expression and PGE2 production.

Collectively, our data show that treatment with homeopathic dilutions of *A. montana* increases mRNA and protein expression of COX-2 in primary cultured mouse chondrocytes and thereby increases PGE2 secretion. These findings support the effectiveness of homeopathic remedies, particularly *A. montana*, *in vitro* and provide a basis for future research. Further studies are required to investigate the effectiveness of *A. montana in vivo* and the underlying mechanism.

REFERENCES

[1] N. Verma, S. K. Tripathi, D. Sahu, H. R. Das & R. H. Das. (2010). Evaluation of inhibitory activities of plant extracts on production of LPS-stimulated pro-inflammatory mediators in J774 murine macrophages. *Molecular and Cellular biochemistry 336,* 127-135.

[2] C. Lass, M. Vocanson, S. Wagner, C. M Schempp, J. F. Nicolas, I. Merfort &, S. F. Martin. (2008). Anti-inflammatory and immune-regulatory mechanisms prevent contact hypersensitivity to Arnica montana L. *Experimental Dermatology 17*, 849-857.

[3] P. P. Alfredo, C. A. Anaruma, A. C. Pião, S. M. João & R. A. Casarotto (2009). Effects of phonophoresis with Arnica montana onto acute inflammatory process in rat skeletal muscles: an experimental study. *Ultrasonics 49,* 466-471.

[4] F. Wiegant & R. Van Wijk. (2010). The similia principle: Results obtained in a cellular model system. *Homeopathy 99*, 3-14. 2010.

[5] Y. H. Huh, M. J. Kim & M. G. Yeo. (2013). Homeopathic Rhus toxicodendron treatment increased the expression of cyclooxygenase-2 in primary cultured mouse chondrocytes. *Homeopathy 102,* 248-253..

[6] A. P. Kawakami, C. Sato, T. N. Cardoso & L. V. Bonamin. (2011). Inflammatory Process Modulation by Homeopathic Arnica montana 6CH: The Role of Individual Variation. *Evidence-Based Complementary and Alternative Medicine 11,* 12.

[7] C. R. Patil, A. D. Rambhade, R. B. Jadhav, K. R. Patil, V. K. Dubey, B. M. Sonara & S. S. Toshniwal. (2011). Modulation of arthritis in rats by Toxicodendron pubescens and its homeopathic dilutions. *Homeopathy 100,* 131-137.

[8] B. Hinz & K. Brune. (2002). Cyclooxygenase-2－10 Years Later. *Journal of Pharmacology and Experimental Therapeutics 300*, 367-375.

[9] C. S. Williams, M. Mann & R. N. DuBois. (1999). The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene 18,* 7908-7916.

[10] A. W. M. Cheng, T. V. Stabler, M Bolognesi, & V. B. Kraus. (2011). Selenomethionine inhibits IL-1β inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) expression in primary human chondrocytes. *Osteoarthritis and cartilage /OARS, Osteoarthritis Research Society 19,* 118-125.

[11] A. C. Abrahao, R. M. Castilho, C. H. Squarize, A. A. Molinolo, D. D. Santos-Pinto & J. S. Gutkind. (2010). A role for COX2-derived PGE2 and PGE2-receptor subtypes in head and neck squamous carcinoma cell proliferation. *Oral oncology 46,* 880-887.

[12] Y. H. Huh, J.-H. Ryu & J. S. Chun. (2007). Regulation of Type II Collagen Expression by Histone Deacetylase in Articular Chondrocytes. *Journal of Biological Chemistry 282,* 17123-17131.

[13] F. Denizot & R. Lang. (1986). Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods 89*, 271-277.

[14] D. E .Trentham, A. S. Townes & A. H. Kang. (1997). Autoimmunity to type II collagen an experimental model of arthritis. *The Journal of Experimental Medicine 146*, 857-868.

[15] D. A.Willoughby, A. R. Moore & P. R. Colville-Nash. (2000). COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. *The Lancet 355*, 646-648.

[16] H. K. Oh, E. Y. Do & H. R. Park. (2015). Convergence Studies of NO Homeostasis in Cellular Signalling.

Journal of Digital Convergence, 13(12), 461-467.

[17] A. Y. Jang, Y. C. Sueng & J.G. Ji. (2016). The comparative study on physiological activity of White ginseng, Red ginseng and Black ginseng extract. *Journal of Digital Convergence, 14(5)*, 459-471.

[18] B. Hinz, K. Brune & A. Pahl.. (2000). Prostaglandin E2 upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications 272,* 744-748.

김 윤 규(Kim, Yun Kyu) [정회원]

- 2009년 2월 : 우석대학교 생명공학과(이학사)
- 2011년 8월 : 전북대학교 해부학과(이학석사)
- 2012년 8월 ~ 현재 : 서울아산병원 연구원

- 관심분야 : 재생의학, 면역학
- E-Mail : kingsaga@naver.com

여 명 구(Yeo, Myeong Gu) [정회원]

- 1989년 2월 : 조선대학교 유전공학과(이학사)
- 1991년 2월 : 조선대학교 유전자과학과(이학석사)
- 1995년 2월 : 조선대학교 유전자과학과(이학박사)

- 2011년 3월 ~ 현재 : 남부대학교 통합의료학과 교수
- 관심분야 : 세포신호전달계, 세포이동
- E-Mail : mgy11@nambu.ac.kr