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Therapeutic effects of PG201, an ethanol extract from herbs, through cartilage protection on collagenase-induced arthritis in rabbits

Kyoung Chul Park [a,1], Eun Jin Park [d,1], Eun Ran Kim [a], Yeonran Kim [a], Suk Hee Chung [b], Byoung Wook Cho [c], Sunyoung Kim [a,d], Mirim Jin [a,*]

[a] PanGenomics, Co. Limited, Biotechnology Incubating Center, Seoul National University, Seoul 151-742, Republic of Korea
[b] Department of Oriental Rehabilitation Medicine, KyungHee University Medical Center, Seoul 130-701, Republic of Korea
[c] ChumSeongDae Oriental Medicine Clinic, Seoul 134-202, Republic of Korea
[d] School of Biological Science, Institute of Molecular Biology and Genetics, Seoul National University, Republic of Korea

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Abstract

In order to assess the therapeutic effects of PG201 (an ethanol extract from herbs) on osteoarthritis, we investigated whether PG201 could suppress the disease progression of collagenase-induced arthritis (CNIA) in rabbits. The right knees of rabbits were injected intra-articularly with collagenase, and the rabbits were orally treated with distilled water (DW), PG201 (200 mg/kg) or diclofenac (DCF, 10 mg/kg) once a day for 8 weeks. Oral administration of PG201 significantly suppressed the stiffness and bone space narrowing. Cartilage erosion and GAG release ($p < 0.01$) were considerably reduced in the knee joints. As well, the mRNA expression of matrix degradation enzymes including MMP-1, -3, and -13 was decreased. On the contrary, the concentrations of TIMP-2 in the synovial fluids were considerably amplified in the PG201 treated group ($p < 0.01$), but not in the DCF treated group. The pathologic inflammatory molecules involved in cartilage destruction such as IL-1β, PGE_2, and NO were also diminished by PG201. Taken together, these results indicate that PG201 has therapeutic effects on CNIA through the prominent protection of cartilage. PG201 indeed has great potential as a form of treatment for osteoarthritis.
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Osteoarthritis (OA) is a degenerative disease characterized by progressive articular cartilage destruction in multiple joints. The initiation of cartilage breakdown is stimulated with mechanical stress or injury, and the degenerative processes slowly progress over many years [1]. The earliest histological pathology consists of fibrillation or fissuring of the cartilage surface. However, complete erosion of the cartilage matrix, which is the major characteristic feature of arthritis, penetrates down to the subchondral bones. In the advanced stage of OA, the abnormal remodeling of cartilage and subchondral bone results in the formation of osteophytes at the joint surface and margins, which irreversibly destroys the affected joint [2].

Particularly, previous studies have shown that matrix metalloproteinases (MMPs) and their tissue inhibitors play important functions in cartilage matrix turnover [3]. Matrix metalloprotease binds to the tissue inhibitors of matrix metalloprotease (TIMPs) on a 1:1 basis by forming high affinity complexes [4]. However, in OA subjects, up-regulated MMPs, which are elevated to a much greater extent than TIMPs, are considered to critically contribute to the degradation of extracellular matrix [5,6]. Proinflammatory cytokines such as IL-1β,

* Corresponding author. Fax: +82 2 873 8022.
E-mail address: mirimj@hanmail.net (M. Jin).
[1] These authors contributed equally to this study.

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produced by activated synoviocytes or cartilage itself directly or through other mediators such as PGE_2 and NO, induce the biosynthesis and secretion of MMPs [7–9]. Conversely, it is reported that amounts and activities of TIMPs are lowered in the synovial fluid of knees with severe osteoarthritis [10]. The imbalance ratio of TIMPs to MMPs results in the continued destruction of proteoglycan and collagen of cartilage in osteoarthritis, much like in the case of rheumatoid arthritis [11,12]. Protection against cartilage destruction is the ultimate goal for oasteoarthritis treatment; however, current pharmacological therapies remain unsatisfactory [13,14].

PG201 is an ethanol extract from 12 herbs and its safety is recognized. PG201 has been developed on the basis of the known function of each herb for arthritis treatment. We previously reported that PG201 demonstrates strong cartilage protective effects by control of key components in RA pathogenesis including the down-regulation of proinflammatory cytokines and the up-regulation of anti-inflammatory cytokine IL-4, as well as in the ratio of TIMP to MMP [15]. Using a rabbit experimental model of OA, we investigated whether PG201 has protective effects on cartilage degradation in collagenase-injected knee joints. X-ray radiography and histological data of stifle joints were obtained, and molecular pathologic indicators involved in cartilage destruction such as MMPs, TIMPs, and inflammatory mediators were analyzed. Here, we show that PG201 has great potential for use in general therapeutics as an anti-inflammatory and cartilage protective agent in OA.

Materials and methods

Preparation of PG201. PG201 was prepared using the same protocol previously described [15]. Briefly, all herbs (for detail, see [15]) used were purchased from a market specializing in herbs (Kyungdong herb market, Seoul, Korea). The moisture content of each herb was less than 10% of its overall weight. The herbs (total 70 g as dry weight) were mixed, minced by grinder (Rong Tong Iron Works, Taiwan), and extracted by storing in a 1 L liquid mixture of 25% ethanol for 3 months at 4 °C. The supernatant was filtered with 10-μm cartridge paper, the ethanol was removed by rotary evaporation (Eyela, Japan), and concentrated extracts were freeze-dried. This scale generally obtains 10 g of brown powder.

Chemicals. Collagenase (*Clostridium histolyticum*, type II; enzyme activity 456 U/mg) was purchased from Sigma (St. Louis, MO); diclofenac sodium (DCF) from Health Eagle (China); TRIzol from GibcoBRL (Gaithersburg, MD); AMV RT kit from Takara (Kyoto, Japan); expanded high fidelity PCR system from Roche (Mannheim, Germany); SBYR Green master mix from PE Applied Biosystems (Warrington, UK); TIMP-2 ELISA kits from Endogen (Cambridge, MA); Glucosaminoglycan (GAG) assay from Biocolor (Belfast, N Ireland, UK); PGE_2 and NO assay from R&D system (Minneapolis, MN).

Induction of collagenase-induced arthritis and drug treatment. Twenty New Zealand white male rabbits were obtained from the Animal Experimental Center at Seoul National University (Seoul, Korea) and individually housed (60 cm × 60 cm × 40 cm). All animal experiments in this study complied with the standards set forth in the University Animal Care and Use Committee guidelines established by Seoul National University. Rabbits weighing 2.5–3 kg (aged 9–10 weeks) at the start of experiments (day 1) were anesthetized with an intra-muscular injection of 45 mg/kg ketamine hydrochloride (KETAMINE 50, Yuhan, Korea) and 2 mg/kg xylazine (Rompun, Bayer, Korea). The shaved right knee joints of all rabbits were injected intra-articularly with 250 μl collagenase solution (4 mg/ml) or saline as a naïve control. The same collagenase injection procedure was applied once more on day 4 according to the method of Kikuchi et al. [16]. Following the initial injection of collagenase (day 1), the rabbits were divided into three groups ($n = 16$ per group). For 8 weeks, control groups were orally treated with 200 μl distilled water (DW) or diclofenac sodium (10 mg/kg) suspended in DW, respectively, and the other group was orally treated with 200 μl PG201 at the concentration of 200 mg/kg on a daily basis. This concentration was previously determined based on the results from dose–response experiments (data not shown). Stiffness of knees, radiological and histological changes of cartilages were examined, and levels of inflammatory mediators were measured at indicated time points during the 8-weeks period.

Macroscopic scoring of stiffness in knee joints. The assessment of stiffness was scored according to the Health Assessment Questionnaires disability score with some modification [17,18]. The movement of knees in naïve rabbits without collagenase injection was observed and compared with that in rabbits injected with collagenase. 0 indicates "without any difficulty," 1 indicates "with some difficulty," 2 indicates "with much difficulty," and 3 indicates "unable to do." Scoring was performed by at least three independent veterinarians without prior knowledge of the experimental groups.

Radiographic examination. Radiographs of knee joints were taken to the Veterinary Medical Teaching Hospital, College of Veterinary Medicine, Seoul National University. A specialist in veterinary radiology using standard exposure techniques took a cranial and caudal view as well as a lateral view of each knee joint at 4 and 8 weeks. Each knee radiograph was graded 0–4 according to the Kellgren and Lawrence scale with some modification [19], and sclerosis, joint effusion, and joint space narrowing were used as main criteria for scoring in our experimental system. 0 indicates "normal joint," 1 indicates "subchondral sclerosis or possible osteophytes with a normal joint space," 2 indicates "subchondral sclerosis or joint effusion with possible narrowing of the joint space," 3 indicates "definite narrowing of joint space," and 4 indicates "marked narrowing of the joint space." Osteoarthritis was considered to be present in the knees if the radiographic grade was greater than 2. Five independent veterinarians without prior knowledge of the experimental groups performed the scoring.

Histological examination. At 8 weeks, rabbits were sacrificed for histological examination. Thereafter, the right knee joints were dissected and all soft tissues were removed, then the lateral and medial sides of the femoral condyle and tibial plateau were fixed with 10% neutral buffered formalin (pH 7.4) and decalcified with 8% HCl and 8% formic acid for 5 weeks. They were embedded in paraffin and the four different specimens (7 μm section) from each knee joint were cut with a microtome ($n = 32$ per group). These sections were stained with safranin O for detection of proteoglycan loss in the cartilage. Cartilage depletion was indicated visually by diminished safranin O staining of the proteoglycan matrix, and was scored arbitrarily as 0 when normal or 1–3 according to the degree of depletion (loss of staining). The destruction was graded separately on a scale of 0–3, ranging from fully stained cartilage to distained cartilage or complete loss of articular cartilage [15,20]. All these histological evaluation procedures were performed blind.

Measurement of proteoglycan degradation. Synovial fluids were aspirated from the right knee joint area at 4 and 8 weeks. The samples were assayed for sulfated GAG as a measurement of proteoglycan degradation. The GAG assay is based on the specific binding of the

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