

# Increasing Intrinsic Hyaluronic Acid and Down-regulation of Inflammation Markers in Synovial Fluid from Patients with Knee Osteoarthritis may be Associated with Symptom Relief after Intra-articular Injection of Hyaluronic Acid

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## Research article

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# Abstract

**Background:** Hyaluronic acid (HA) is the most common intra-articular therapy used to treat mild to moderate osteoarthritis (OA). However, the mechanism involved in this treatment is still not fully understood. The aim of the present study was to examine the effect and the possible mechanism of intra-articular HA (IAHA) injection in patients with knee osteoarthritis (OA).

**Methods:** Twenty-eight patients with Kellgren–Lawrence scale II to III were enrolled in this study. All patients underwent ultrasound-guided injection using three consecutive weekly IAHA. Functional ability and pain were determined by the Western Ontario and McMaster University Index (WOMAC) questionnaire and visual analog scale (VAS). Further, the levels of HA, metalloproteinase (MMP)-1, MMP-3, MMP-13, interleukin (IL)-1 $\beta$  and IL-6 in synovial fluid were determined weekly before HA injection.

**Results:** Functional improvement and pain relief were observed 4 weeks after treatment. At week 4, a significant increase of HA concentration was found, and the concentration of inflammatory cytokines including IL-1 $\beta$ , and IL-6, as well as matrix MMP-3 and MMP-13 significantly decreased. However, no significant difference was observed in MMP-1 level.

**Conclusion:** These results suggest that increasing HA accumulation in synovial fluid may be associated with disease relief after weekly IAHA injection in patients with knee OA.

## Introduction

Knee osteoarthritis (OA) is a common worldwide health problem. Epidemiology studies have shown that the occurrence of knee OA in people aged over 65 is more than 20 percent [1]. During OA, the biochemical status impairment leads to the destruction of subchondral bone and joint cartilage. Some studies indicate that synovial fluid (SF), synovium, knee tendon, and ligament were also involved in the pathogenesis and development of OA [2]. In addition, age is one of primary risk factors of OA, since chondrocytes have a reduced capacity to restore and maintain the extracellular matrix in cartilage in elder patients [2].

Pro-inflammatory mediators initiate numerous factor expressions and therefore trigger signal pathways during OA development. Interleukin (IL)-1 $\beta$  induces IL-6 expression, which then enhances osteoclast differentiation and maturation, results in an acceleration of bone absorption. IL-1 $\beta$  also induces the expression of matrix metalloproteinases (MMPs) [3, 4]. In the early stage of OA, matrix metalloproteinase (MMP)-1 (collagenase 1), MMP-8 (collagenase 2) and MMP-13 (collagenase 3) degrade type-1 collagen fiber. The other MMPs, such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B), further degraded the collagen fiber metabolites [5, 6]. In addition, IL-1 $\beta$  enhances cyclooxygenase (COX)-2 expression, which may be associated with pain generation in OA sufferers.

Generally, SF originates from the ultra-filtration through the rich vascular network of the synovial tissue. This network functions as a transport medium for nutritional substances to avascular articular cartilage

and aids in the mechanical function of joints by reducing friction and absorbing shocks to the articulating surfaces [7]. Further, it also supplies oxygen and nutrients and removes carbon dioxide and metabolic wastes. SF enters the cartilage by diffusion and is further dispersed during motion. In healthy knee joints, this fluid is clear, viscid, and found in quantities of less than 3.5 ml. Moreover, SF is able to reflect the basic pathological processes in various diseases that occurred in the joint.

Intra-articular hyaluronic acid (IAHA) injection is a widely used treatment for pain relief and functional ability improvement in OA patients. A previous systematic review has demonstrated that HA possesses the anti-inflammatory, chondroprotective, and analgesic effects [8]. Therefore, HA may be beneficial for treating patients with knee OA. However, the effect of HA on endogenous HA production and pain relief is still unclear. In the present study, we examined the effects of HA injection on disease severity and the levels of HA, MMPs, and pro-inflammatory cytokines production in SF in patients with moderate grade of OA.

## Material And Method

### Patients

All participants provided written informed consent prior to enrolment into the study. All procedures have been approved by the Institutional Review Board of the National Cheng Kung University (NCKUIRB), Taiwan (A-ER-103-139). This prospective study examined the results of the unilateral knee in 28 patients with K-L grade 2 to 3 (11 males and 17 females, aged from 51 to 82-year-old) (Figure 1). All patients consulted for IAHA injection therapy were at least 45 years old and met the American College of Rheumatology (ACR) criteria for knee OA [9]. There were no significant differences in all measured baseline parameters between males and females, detailed description in Table 1. Exclusion criteria were: (1) secondary knee OA following GREES (Group for the Respect of Ethics and Excellence in Science) criteria; (2) a history of knee surgery; and (3) a history of arthrocentesis and an IA steroid or hyaluronic acid injection 3 months before the study. All patients were evaluated by the same observer using the Western Ontario and McMaster University Index (WOMAC) function subscale OA index, and the Visual Analogue Scale (VAS) score before the IAHA, and at week 1, week 2, and week 4 post first IAHA injection. During the study period, no analgesics except acetaminophen were allowed. Moreover, any analgesics were not allowed for 72 h preceding the clinical assessment [10]. Further, the person who did the evaluations was not involved in the IAHA injection to patients.

### IAHA injection

All patient were given three consecutive weekly injection of 2.5 ml HA (ArtiAid, Maxigen Biotech Inc., Taoyuan City, Taiwan or ARTZDispo, Seikagaku corporation, Takahagi-shi, Japan) into the suprapatellar pouch under ultrasound guidance 3 times weekly (Fig. 2, red arrow). Four times of SF aspiration (Fig. 2, green arrow) was performed during a therapeutic period (Figure 2).

## WOMAC

WOMAC was assessed by the investigator before SF aspiration. This questionnaire, first introduced in 1988, is used to assess the health status of patients. It consists of 33 items that evaluate the health and function of the patient from various aspects, including clinical symptoms (5 questions), severity of knee joint stiffness (2 questions), degree of pain (9 questions) and activity of daily living (17 questions). Each question has five subscales where the best situation is scored as never or none and the worst as extreme or always. Here, higher scores are representative of a better health situation and less pain.

## VAS

Each patient was administered the VAS by the investigator before SF aspiration. A 100 mm horizontal line served as the VAS and separated the description of “no pain”, graphed as a smiley face, from the other description of “extreme pain”, graphed as a crying face. All patients involved in this study chose scores by free-will without any influence from the investigator.

## Ultrasound-guided SF aspiration

SF was aspirated from each study participant by the same investigator under ultrasound guiding [11] and transported immediately to the laboratory in an icebox at 4°C. The samples were centrifuged for 20 min at 10000 x g, after which the precipitated pellet was discarded and the supernatant pipetted into 2.0 ml Eppendorf tubes and stored at -80°C until analysis.

## Determining HA concentration

The HA concentrations of the SF samples were determined by a modified micro-method of the uronic acid assay according to the Morgan-Elsion method [12]. The diluted SF samples were boiled with a sulfuric acid/borax mixture, and after the addition of m-phenylphenol, a pink color developed. The color intensities were then determined in a microplate reader with 560 nm and compared with those of known standard concentrations of HA. All assays of each sample were performed in triplicate to reduce error, the resulting means of which were used for analysis.

## Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-1 $\beta$ , IL-6, as well as MMP-1, MMP-3, and MMP-13 in the SF were determined by an ELISA detection kit (R&D system, USA) with all procedures following the manual's instructions. Briefly, a 96-well microplate was coated with a capture antibody overnight then washed by phosphate buffered saline with Tween 20 (PBST) for 3 times. All samples were digested by 4 mg/ml hyaluronidase in a 1: 1 mix, and then incubated at 37°C for one hour [13]. 100  $\mu$ l of 10-fold diluted SF was loaded into the wells

in duplicate and allowed to sit for 2 hours at room temperature. After washing three times, the detection antibody was added and incubated at room temperature for one hour. After washing again, 3,3', 5,5'-tetramethylbenzidine (TMB) chromogen was added and allowed to incubate for 30 minutes. Subsequently, 2% sulfuric acid was added into the wells to stop the chromogenic reaction and measured the absorbance at 405 nm using a plate reader.

## Statistical analysis

Data were expressed as the means  $\pm$  standard deviation (SD). The paired sample *t*-test analysis was used to make pairwise comparisons between the groups. Statistical significance was set at  $p < 0.05$ .

## Results

### Effects of IAHA injection on pain and function ability

Under the questionnaire analysis, the visual analog scale (VAS) score was significantly reduced one week after IAHA injection ( $75.2 \pm 14.7$  vs.  $44.2 \pm 17.2$ ,  $p < 0.05$ , Figure 3A) and kept decreasing throughout the entire therapeutic period. In addition to the decreasing VAS scores, the WOMAC scores also decreased starting the 2<sup>nd</sup> week after IAHA injection ( $45.04 \pm 11.9$  vs.  $24.2 \pm 10.6$ ,  $p < 0.05$ , Figure 3B).

### Effect of IAHA injection on HA concentration in SF

The HA concentration in the SF increased gradually during the HA injection therapeutic period and reached a significant difference after the 4<sup>th</sup> week of therapy ( $0.41 \pm 0.1$  vs.  $0.78 \pm 0.3$ , Figure 4A) compared with pre-treatment.

### Effects of IAHA injection on IL-1 $\beta$ , IL-6 and MMP expression in the SF

The IL-1 $\beta$  in the SF was significantly decreasing after the IAHA injection during the therapeutic period ( $13.3 \pm 11.5$  vs.  $4.1 \pm 5.1$  pg/ml,  $p < 0.05$ ;  $2.5 \pm 2.9$  pg/ml,  $p < 0.05$ ;  $2.1 \pm 2.0$  pg/ml,  $p < 0.01$ , in pre-treatment vs. 1<sup>st</sup>, 2<sup>nd</sup> and 4<sup>th</sup> weeks respectively) (Figure 4B). As shown in Figure 5A, the IL-6 concentration in the SF significantly decreased in the 4<sup>th</sup> week after IAHA injection ( $495.4 \pm 471.7$  vs.  $53.82 \pm 38.5$ ,  $p < 0.05$ ) compared with pre-treatment. The MMP-3 concentration in the SF started decreasing at the 2<sup>nd</sup> week after IAHA injection ( $685.7 \pm 393.3$  vs.  $261.8 \pm 163.9$ ,  $p < 0.05$ ) (Figure 5C), while the MMP-13 concentration significantly decreased at the 4<sup>th</sup> week after IAHA injection ( $75.6 \pm 85.4$  vs.  $7.7 \pm 14.5$ ,  $p < 0.05$ ) compared with pre-treatment (Figure 5D). Unlike MMP-3 and -13, the MMP-1 concentration showed no significant difference during therapy (Figure 5B).

## Discussion

In the present study, we have demonstrated that IAHA injection exerted a significant effect in pain relief and functional improvement in patients with knee OA. Decreased IL-1 $\beta$  and increased HA levels in SF were observed after the 3<sup>rd</sup> round of IAHA injections. Further, IAHA injection significantly decreased the levels of MMP3 and MMP-13, but not MMP-1, in SF compared with that in pre-treatment groups. We suggested that four-week IAHA injection may decrease disease severity of OA by increasing HA generation as well as decreasing proinflammatory cytokine and MMPs production in patients with knee OA, at least partially.

IAHA injection therapy is a commonly used current treatment for restoring the sufficient concentration of HA in patients with knee OA [8, 14]. Ultrasound is a noninvasive procedure with no radiation and almost no discomfort for patients and is useful in diagnosing joint synovitis and SF effusion in the knee [15]. In this study, we employed ultrasound-guided needle placement not only to completely aspirate knee SF but also to deliver the HA more precisely. Our treatments have shown that the concentration of HA in the SF increased gradually and reached a significant difference after the 4<sup>th</sup> week of therapy. Previous studies suggest that disappearance of HA is multiphasic with very slow terminal clearance, while other studies indicate that the half-life of HA is around 24 h [16]. It is likely that the increased HA concentration in patients' knee joint one week after extrinsic HA injection may be a result of the extrinsic HA accumulation and/or intrinsic HA generation. However, more investigations will be needed to clarify this.

Suppressing IL-1 $\beta$ , MMP-3, and MMP-13 expressions may be involved in the increase of HA concentration in SF. IL-1 $\beta$  can trigger the elevation of hyaluronidase mRNA followed by the degradation of HA in cultured synovial membrane cells [17]. Elevated hyaluronidase activity results in a reduced concentration and decreased molecular weight of HA in SF of knee OA [18]. On the other hand, injecting MMP-13 siRNA into the knee joint can delay joint cartilage degradation in a mouse knee OA model [19]. Low molecular weight HA produced from the degradation of HA by hyaluronidase promotes MMPs expression [20, 21]. In contrast, IAHA decreased MMP-3 and MMP-13 expression after 2 rounds of therapy. Further, up-regulating MMPs not only cause joint cartilage degradation but also activate other MMP proteins. In the present study, IAHA injections significantly decreased the IL-1 $\beta$ , MMP-3, and MMP-13 expressions in SF. We suggested that the inhibition of IL-1 $\beta$ , MMP-3, and MMP-13 expression after IAHA injections may play an important role in HA production in SF.

Three consecutive week injections of IAHA may be effective in attenuating disease severity and inflammatory response in knee OA patients. Although 3-5 weekly injections are presently the most widely adopted therapeutic strategy for knee OA treatment, the IAHA injection regime remains undetermined (Table 2). In the present study, IAHA inhibited IL-1 $\beta$  production and affected the metabolism of HA, which resulted to an increase in the HA production, in SF after the 3<sup>rd</sup> treatment. These may account for the clinical observation of pain relief and improved functional ability. In summary, three consecutive week injections of IAHA may attenuate disease severity in knee OA patients. Further, decreased pro-

inflammatory cytokines, MMP-3, and MMP-13, as well as increased HA levels in SF may be involved in IAHA-exerted effects against OA.

Despite, there were several limitations in this study. Serial intra-articular aspirations from osteoarthritic knee with placebo (normal saline) injection in clinical practice is unethical; therefore, we did not enroll any patient as the control group. Further, the sample size in this study was small because our patient populations were dominant in mild to moderate knee osteoarthritis with K-L grade 2 to 3, which did not meet our inclusion criteria. Therefore, a future study with bigger sample size and long-term follow-up will be needed.

## **Conclusion**

Increasing HA accumulation and down-regulation inflammation markers in SF may be associated with disease relief after three doses of HA injection in knee osteoarthritis patients.

## **Abbreviations**

HA, hyaluronic acid; OA, osteoarthritis; SF, synovial fluid; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay; WOMAC, Western Ontario and McMaster University Index; VAS, visual analog scale; IL, interleukin; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; ACR, American College of Rheumatology; GREES, Group for the Respect of Ethics and Excellence in Science; US, ultrasound US.

## **Declarations**

## **Ethics approval and consent to participate**

Ethical approval: All procedures were approved by the Institutional Review Board of National Cheng Kung University Hospital (NCKUH)

IRB No.: A-ER-103-139

Informed consent: Written informed consent was obtained from all patients included in this study.

## **Consent for publication:**

Non-applicable

## **Availability of data and material:**

The datasets used during the current study are available from the corresponding author on reasonable request.

## Competing interests:

The authors declare that they have no competing interests

## Funding:

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## Author contribution:

JHY and IMJ: study conception and design

PYK, CJS, KCW, TCC, and IMJ: acquisition of data

JHY, PYK, CJS, KCW, TCC, CYY, and IMJ: analysis and interpretation of data

JHY, PYK, CYY, and IMJ: drafting of the article

All authors have read and approved the final submitted manuscript.

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## Tables

Table 1 and Table 2 is not available in this version of the manuscript.

## Figures

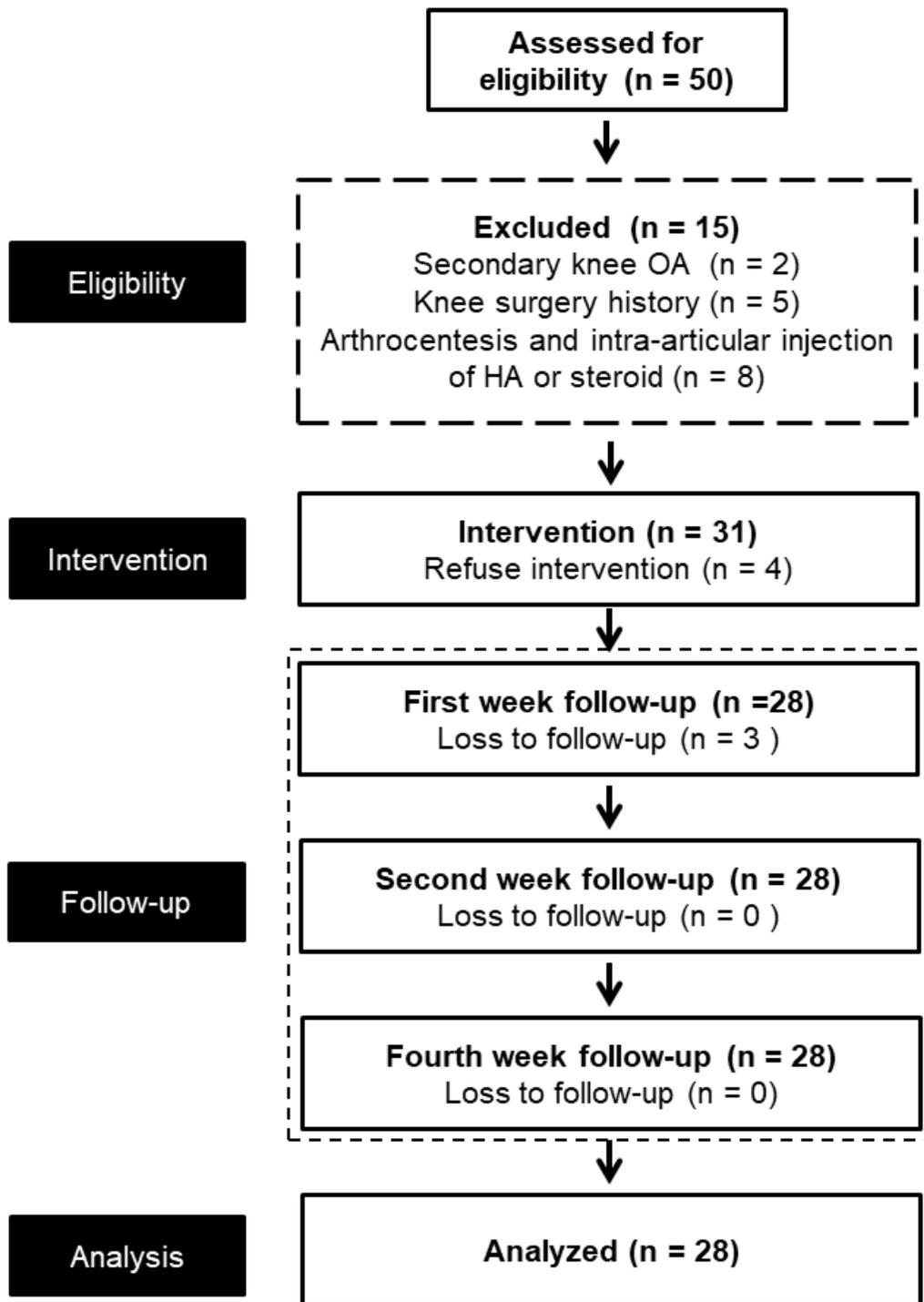
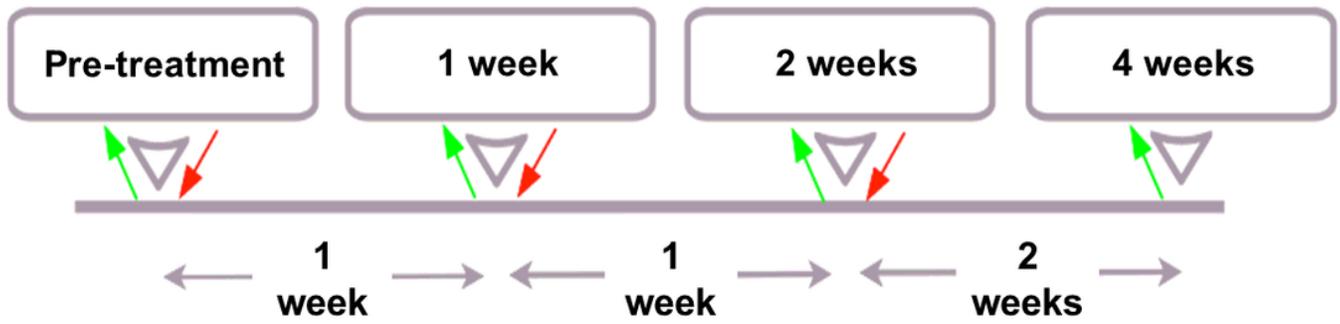


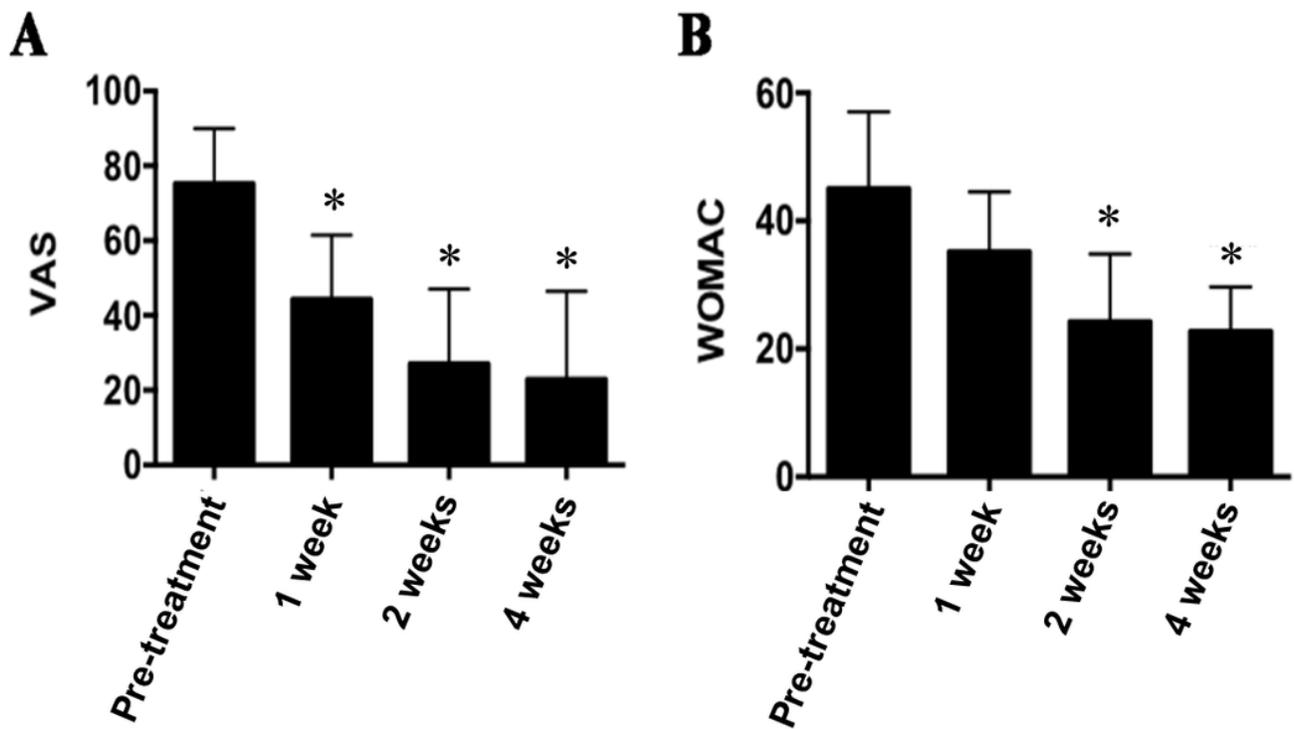
Figure 1

The consort diagram of the present study.



**Figure 2**

Study flow chart. Four SF aspirations were taken in this study at week 0, 1, 2, and 4. The green arrow indicated SF aspiration before a red arrow, the IAHA injection. After 4 weeks of therapy, the participants were recruited for final SF aspiration.



**Figure 3**

IAHA injection decreased the VAS and WOMAC scores in osteoarthritis patients. Both VAS (A) and WOMAC (B) scores were assessed at week 0, 1, 2, and 4 after IAHA injections. The data are mean  $\pm$  SD. \* $p < 0.05$  compared with pre-treatment group.

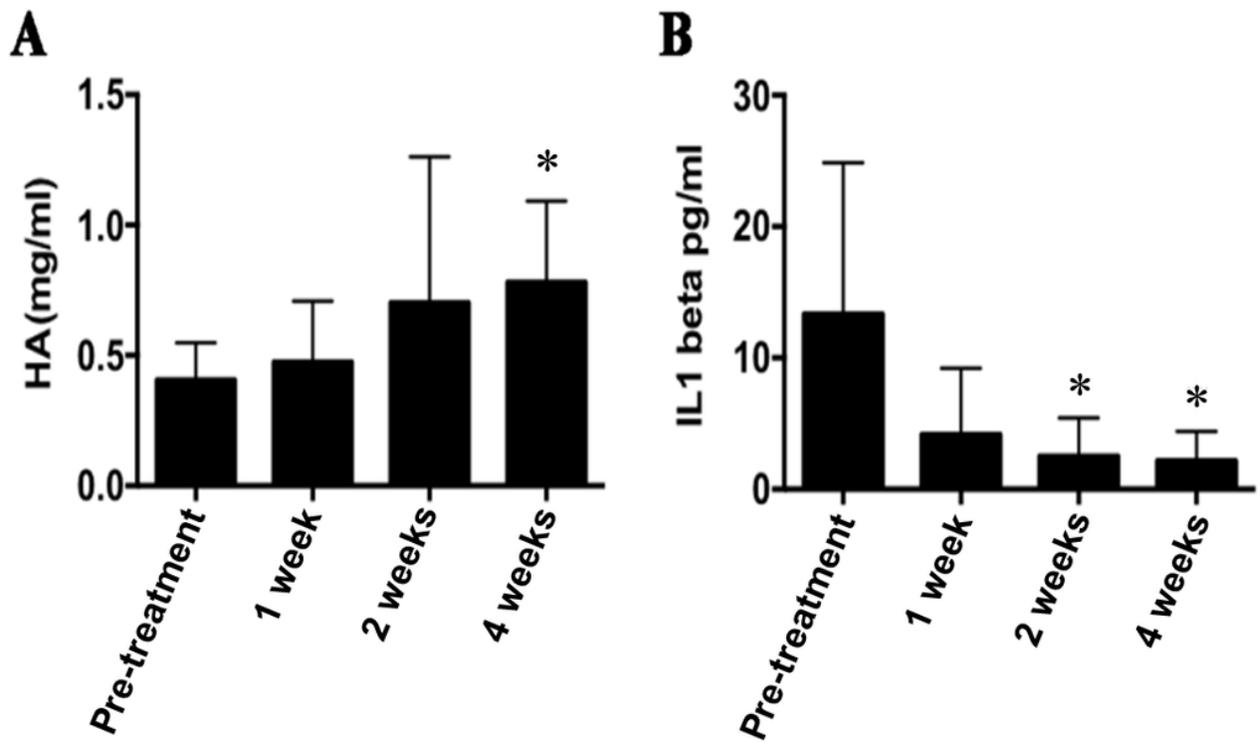
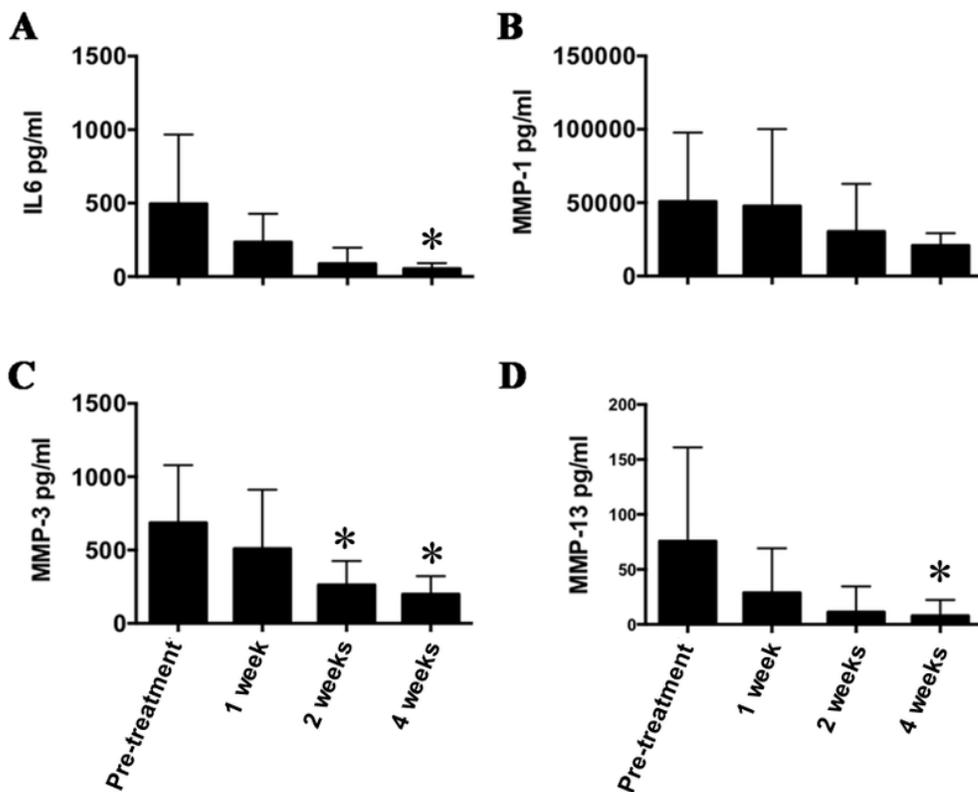


Figure 4

Increased HA production (A) and decreased IL-1 $\beta$  concentration (B) in SF were presented in osteoarthritis patients after IAHA injection. The data are mean  $\pm$  SD. \* $p < 0.05$  compared with pre-treatment group.



## Figure 5

The concentrations of IL-6 (A), MMP-1 (B), MMP-3 (C) and MMP-13 (D) were measured in the synovial fluid of osteoarthritis patients after IAHA injection. The data are mean  $\pm$  SD. \* $p < 0.05$  compared with pre-treatment group.