Research article

Study design: Intraarticular injection of hyaluronan versus saline in the treatment of adjuvant-induced arthritis: a randomized controlled trial

Hyaluronan Modulates Accumulation of Hypoxia-Inducible Factor-1 alpha, Inducible Nitric Oxide Synthase, and Matrix Metalloproteinase-3 in the Synovium of Rat Adjuvant-Induced Arthritis Model

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Abstract

Introduction: Hypoxia is a feature of the inflamed synovium in rheumatoid arthritis (RA). Intra-articular injection of hyaluronan (HA) may be considered a potential way to treat RA. However, the exact molecular mechanism of HA on decreased cellular responses to hypoxic environment is unclear. The present study has been designed to use the adjuvant-induced arthritis model to examine the effects of HA on the changes of immunohistochemical expressions of hypoxia-inducible factor-1α (HIF-1α), inducible nitric oxide synthase (iNOS), and matrix metalloproteinase-3 (MMP3) in the synovial tissues at the early phase of arthritic inflammation.

Methods: Monoarthritis was induced in adult male Sprague-Dawley (250–300 gm) via intraarticular injection of complete Freund's adjuvant (CFA) into the tibiotarsal joint. The CFA-induction arthritis animals were divided into three groups: treatment (intraarticular injection of HA), placebo (intraarticular injection of saline) and controls (no treatments). Functional evaluations of edema and pain behavior, histology, and HIF-1α, iNOS, and MMP3 immunohistochemistry were performed before, after the first injection, three injections, and on the follow-up injection of the treatments.

Results: Intra-articular injection of HA also significantly suppressed the mechanical allodynia ($p < 0.001$) and overexpressions of HIF-1α ($p < 0.001$), iNOS ($p = 0.004$) and MMP3 ($p < 0.001$) immunoreactivity in synovium.

Conclusions: This study demonstrated that early intervention of HA is an effective protection against accumulation of inflammation-induced HIF-1α, iNOS, and MMP3 to limit erosive damage in CFA-induced model of arthritis.

Introduction

Hypoxic microenvironment is a hallmark of the inflamed synovium and its importance in the pathogenesis of rheumatoid arthritis (RA) has been documented [1-4]. In human and animal arthritis models, the importance of hypoxia for the development and persistence of RA has been demonstrated [1, 5]. Previous studies have demonstrated the hypoxic nature of the synovium of patients with RA and the constitutive expression of hypoxia-inducible factor-1α (HIF-1α), a key regulator of hypoxia transcriptional response. In RA joints, it has been shown to express increased presence and accumulation of HIF-1α and HIF-1 target genes in synovial lining cells and articular chondrocytes under hypoxic condition, which aggravates joint inflammation [6, 7]. Previous studies also demonstrated that hypoxia takes place in synovium at the pre-arthritic stage or early stage of the disease and have a close spatial relationship and positive severity correlation with synovitis [8]. Therefore, HIF-1α is identified to be a key player in the pathogenesis of RA and a potential therapeutic target in RA development.

Nitric oxide (NO) synthesized from arginine by nitric oxide synthases (NOS), is an important chemical mediator of inflammation. The inducible isoform of NOS (iNOS) is primarily responsible for producing large amounts of NO and its overexpression has been linked with the progressive inflammation and tissue destruction observed in hypoxic

70 experimental arthritis [9, 10] and human rheumatoid synovium [11, 12]. Matrix
71 metalloproteinases (MMPs), the most important matrix-degrading enzymes in RA, act as key
72 mediators of the resorption of cartilage, bone, synovial fluid, and adjacent soft tissue, which
73 occurs as part of the pathological destruction of joint tissue [13]. Among dozens of MMPs,
74 MMP3 (stromelysin 1) has been reported to be the major enzyme produced by fibroblasts and
75 macrophage-like cells in the synovium, and that the level of MMP3 is significantly higher in
76 synovial fluids from patients with RA [14-16]. Based on previous studies, under the
77 inflammatory conditions of RA, the levels of HIF-1α, iNOS, and MMP3 are significantly
78 higher in synovial fluids and implicated in the pathogenesis of RA. Expressions of iNOS and
79 MMP3 are probably regulated by HIF-1α in the cellular response to hypoxic and
80 inflammatory environments [11, 17, 18]. Therefore, inhibition and/or down-regulation of
81 these molecules may exert anti-hypoxic and anti-inflammatory effects.
82 Hyaluronan (HA) is a polymer of disaccharides, which has high capacity for holding
83 water and possesses high viscoelasticity [11]. The intra-articular supplementation of HA can
84 replace synovial fluid, which has lost its viscoelastic properties. HA has been widely used for
85 the treatment of osteoarthritis (OA) [19]. In addition to its characteristic as a lubricating agent,
86 exogenous administration of HA can suppress expression of inflammatory cytokines, MMPs
87 and free oxygen radicals to reduce inflammation in a postlaminectomy rat model [20] and
88 patients with RA [21]. Therefore, it has been expected that the intra-articular injection of HA

is more efficacious in treating RA, which principally characterizes articular synovitis [21, 22]. However, its clinical use for RA joint treatment is still rare because the immunoregulatory action of HA is still debatable.

Complete Freund's adjuvant (CFA)-induced arthritis shares some characteristics of RA. This model mirrors much of the pathology of RA including hyperplasia of the synovial tissues, inflammatory infiltration of the joints, and destruction of bone and cartilage in the synovial joint [23]. The present study has been designed to use the adjuvant-induced arthritis model to examine the effects of HA on the changes of immunohistochemical expressions of HIF-1α, iNOS, and MMP3 in the synovial tissues at the early phase of arthritic inflammation. We hypothesize that addition of HA will alleviate inflammatory nociception and impede the accumulation of arthritis-induced HIF-1α, iNOS, and MMP3 productions at the early phase of the experimental arthritic inflammatory joint. If this hypothesis is correct, it will offer at least a partial explanation for efficacy of topical HA application in the subsequent inhibition of hypoxic inflammation in this preclinical model.

Materials and methods

General design

Arthritis was induced arthritis on all animals by intra-articular injection of CFA. After a day of CFA induction, the arthritic animals were randomly divided into three groups (n = 90) according to three treatments named as: (1) Control (sham injection by needling, intra-articularly manipulated and no solution administration, No-tr group, n = 30); (2) Placebo (50 µl saline intra-articularly administered, SA group, n = 30); (3) Treatment (50 µl HA intra-articularly administered, HA group, n = 30). Injections of HA or saline were given every 2 d (Days 2, 4, and 6). The evaluation instruments were edematous swelling of the paw, pain behavioral assessments, histology, and immunohistochemistry. Assessments were performed at pre-arthritic (Day 0), post-arthritic (Day 1) and 3 h after the treatment of one injection (1 dose, 1D) on Day 2, three injections (three doses, 3D) on Day 6, and follow-up 6 d after 3D (3D6d) on Day 12. The flow diagram is presented in figure 1.

Animal preparation

Ninety adult male Sprague-Dawley (CD®(SD) IGS BR; purchased from BioLASCO Taiwan Co., Ltd.) rats weighing 250–300 g were kept in the Laboratory Animal Center of China Medical University. Effort was made to minimize discomfort and to reduce the number

of animals used. All animal experiments were conducted with the procedure approved by the Animal Care and Use Committee of a university in accordance with the Guidelines for Animal Experimentation.

Induction of monoarthritis

Monoarthritis was induced by an injection of CFA into the unilateral ankle articular cavity. The rats were briefly anesthetized with 4% isoflurane (AERRANE, Baxer Healthcare Corporation, Puerto Rico). A 28-gauge needle was vertically inserted distally into the articular cavity from the gap between the tibiofibular and tarsus bone. CFA with volume of 50 µl (10 mg mycobacterium, F5881, Sigma, MO) was then injected. The monoarthritic animals were separately placed in clear acrylic containers (10½" W × 19" D × 8" H), allowing free movement for at least 24 h to let them adjust to these conditions before any experimentation is performed.

Ultrasound-guided HA injection

Under brief isoflurane anesthesia, ultrasound (Terason t3000™ Ultrasound System, Terason Division, Teratech Corporation, MA, USA) guided injection was performed on the lateral side of tibiotarsal joint, with the transducer in the sagittal plane showing the distal end of tibia and proximal part of the tarsus in the image plane. Needle insertion was

perpendicularly performed to the transducer. HA injection (MW: 1.2–1.4 $\times 10^6$ Da; Ostenil®, 10 mg/ml sodium hyaluronate, TRB CHEMEDICA AG, München, Germany) was documented by recording an image-clip during injection with the needle tip in the image plane.

Pain threshold assessment

The pain thresholds were determined by nociceptive thresholds to mechanical stimulation. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic von Frey anesthesiometer, IITC Inc., CA, USA) adapted with a 0.5 mm^2 polypropylene tip. In a quiet room, the rats were placed in acrylic cages (32 × 22 × 27 cm high) with a wire grid floor for 15 -30 min habituation prior to testing. The polypropylene tip was perpendicularly applied to the central area of the hind paw with with sufficient force to bend the filaments into an “S” shape for 3-4 sec. The test consisted of poking a hind paw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. Testing was initiated with the filament corresponding to 20 log of force (g). The filaments were applied with a gradual increase in pressure until a withdrawal reflex response was finally detected from the animal. The response to this filament is defined if a series of weaker or stronger filaments would be tested. The weakest filament able to elicit a response was taken to be the paw withdrawal threshold (g). The intensity of the pressure was recorded and the

final value for the response was obtained by averaging five measurements.

Measurement of edematous swelling of the paw

The extent of peripheral swelling was assessed by measuring the circumference of the paw at intact and CFA-injected sites with a flexible tape. The paw circumference was obtained by averaging three measurements. The difference in the ankle circumference between the initial value (pre-arthritic data) and that at each time point after injection is expressed as change (%) = 100% × [(post-arthritic circumference) – (pre-arthritic circumference)] / (pre-arthritic circumference). All assessments including paw withdrawal and swelling measurements were performed with the assessor blinded with respect to treatment.

Histology and immunohistochemistry

Animals were killed by anesthetic overdose after treatments of 1D (n = 10 for each group), 3D (n = 10 for each group), and 3D6d (n = 10 for each group) on Days 2, 6, and 12. Hind ankles were collected for histological and immunohistological analysis. The formalin-fixed, paraffin-embedded joint tissues (including synovium and cartilage tissues) were cut at thickness of 5 μm for histology and immunohistochemistry. Histological confirmation of the arthritic pathology was performed with hematoxylin and eosin (H&E)

stained sections. Sections were deparaffinized in 200 ml of Trilogy (Cell Marque Corporation, CA, USA) and incubated with 3% H_2O_2 in methanol for 20 minutes at room temperature. Subsequently sections were treated with proteinase K (Sigma, St. Louis, Mo, USA) at 0.1 mg/mL for 20 min at room temperature to unmask epitopes followed by phosphate buffered saline (PBS) rinse. Sections were incubated with blocking buffer (Power Block^{TM}, Biogenex, USA) for 2 h at room temperature followed by incubation overnight at 4°C with the mouse monoclonal antibody anti- HIF-1α (dil. 1:100, Thermo, CA, USA) and with the following rabbit polyclonal antibodies: anti-iNOS (dil. 1:200, Thermo, CA, USA), anti-MMP3 (dil. 1:200, Abbiotec, CA, USA). After three washes with PBS containing 0.05% Tween-20 for 10 min, sections were incubated with biotinylated anti-rabbit and anti-mouse immunoglobulins (Jackson immunoresearch, PA, USA), followed by a 30 min peroxidase-conjugated streptavidin incubation (Jackson Immunoresearch, PA, USA). Sections were incubated with 3,3′-diaminobenzidine (Biogenex, CA, USA), dehydrated and cover-slipped with Permount (Sigma, NJ, USA). Negative controls were performed by substituting the primary antibody with non-immune serum.

The histopathology of synovium was analyzed by non-parametric scoring system described by Smith et al. (24). The scores ranged from 0 to 3 on the each tissue criteria including intimal hyperplasia, lymphocytic infiltration, subintimal fibrosis and vascularity. The higher aggregate score was considered to reflect increased pathological changes. Five

randomly selected sections were scored and repeated two times for statistical analysis. Quantitative analysis of immunostainings was carried out by light microscopy in synovial tissue lining the joint cavity and synovial tissue attached to the cartilage. The number of HIF-1α, iNOS and MMP3 immunoreactive cells was counted among at least five alternate sections in the more representative fields by using a microscope. Positive nuclei and cytoplasm staining cells for HIF-1α, iNOS, and MMP3 were counted in high-power fields (200× magnification) that contained synovial lining cells. The area sizes of high power fields were calculated by using a stage micrometer (with 100 gradations of 0.01 mm each) when viewed using a 200× objective. Ten fields of each slide were counted for all samples and repeated three times for statistical analysis. Results were expressed as the proportion (%) of number of labeled cells per square millimeter of synovium. For statistical analysis, the mean value obtained from the repeated counts was used. All of scoring and quantitative analyses were assessed by two independent observers who were blinded to the origin of the sections to avoid bias from interobserver variability.

Statistical analysis

The differences of value in each assessment between pre- and post-arthritic evaluations were performed by Student's t-test. The differences among the groups of HA, SA, and No-tr on each dosage (1D, 3D, and 3D6d) were carried out using ANOVA, and later

219 further analyzed by a Bonferroni post-hoc method. Similar statistical analysis methods were
220 used to test the differences among dosages in each group. Non-parametric data (histological
221 synovial scoring) was analyzed using the Kruskal–Wallis test for multiple groups and
222 following Mann–Whitney U-tests for between-group comparisons. Pearson correlation test
223 was applied to study the correlations between pain withdrawal threshold and expressions of
224 immunoreactivities, A p value of <0.05 was considered to be statistically significant. All data
225 was analyzed using SPSS version 10.0 for Windows (SPSS Inc., IL, USA).
226
227

Results

Effect of HA on CFA-induced edema

The serial alterations of the percentage of edema (mean ± SEM) throughout the whole experiment for each group are shown in figure 2A. After a day of CFA-induction, all animals developed severe monoarthritis in the injected paw. There were no significant differences in the non-injected intact paw on circumference among pre- and post-arthritic, and post-treatment conditions for each group ($p > 0.05$, data not shown). The edema of the CFA-injected paw gradually increased, reaching a maximal swelling of 65.51%, whereas there were significant differences on edema between pre- and post-arthritic conditions ($p < 0.001$).

After treatment, the significant time-dependent differences on edema development were observed in each group (HA group: $p < 0.001$; SA group: $p < 0.001$; No-tr group: $p < 0.001$). However, there was no difference in the edema of the arthritic paws among HA, SA, and No-tr groups after treatments of 1D ($p = 0.22$), 3D ($p = 0.41$) and 3D6d ($p = 0.31$). Therefore, intra-articular injections of HA, regardless of different dosages for 1D, 3D, and 3D6d, did not ameliorate joint swelling compared with either SA or No-tr groups.

Effect of HA on CFA-induced inflammatory mechanical nociception

The serial alterations of the paw withdrawal threshold (mean ± SEM) throughout the whole experiment for each group are shown in figure 2B. The mean threshold was 25.07 ± 4.68 g at pre-arthritic condition. However, after CFA-induction, it decreased to 9.32 ± 3.16 g. There was significant difference with pre-arthritic condition ($p < 0.001$).

The significant differences on paw withdrawal threshold were shown among HA, SA, and No-tr groups after treatment of 1D ($p = 0.008$), 3D ($p < 0.001$), and 3D6d ($p <0001$). Significantly lower threshold existed after treatment of 1D, 3D, and 3D6d in SA and No-tr groups compared with those in HA groups (HA vs. SA, $p = 0.04$; HA vs. No-tr, $p = 0.01$ for 1D; HA vs. SA, $p < 0.001$; HA vs. No-tr, $p < 0.001$ for 3D; HA vs. SA, $p < 0.001$; HA vs. No-tr, $p = 0.001$ for 3D6d). The analysis also showed that there was significantly lower threshold found in No-tr group compared with SA group after treatment of 3D ($p =0.03$) and 3D6d ($p = 0.01$). However, no significant difference was observed between these groups after treatment of 1D ($p =1.0$).

There were significant difference among three dosages in HA group ($p < 0.001$), but not in both SA ($p = 0.84$) and No-tr ($p = 0.56$) groups. After HA treatment, the paw withdrawal threshold showed significant increase in 3D and 3D6d treatments compared with 1D treatment (1D vs. 3D, $p < 0.001$; 1D vs. 3D6d, $p < 0.001$). However, no difference was observed between the 3D and 3D6d of HA treatments ($p = 0.05$).

Histopathological assessments

Widening of the synovial cavity, infiltration of inflammatory cells, thickening of the synovial membrane, a narrowing of the synovial space, disruption of the cartilaginous tissue, and bone erosion were apparent in control rats of No-tr group (figures 3A, 3a) and SA group (figures 3B, 3b). The tibiotarsal joints of rats treated with 1D, 3D, and 3D6d of HA were less inflamed, as revealed by decreased number of inflammatory cells, synovial membrane thickening and cartilage destruction (figures 3C, 3c). There were significant differences in lymphocytic infiltration and aggregate score of non-parametric criteria observed among ankle joint synovium from HA and SA and No-tr groups treated with 1D, 3D, and 3D6d (Table 2, $p < 0.05$). Lymphocytic infiltrations in synovium were significantly reduced after HA treatment when compared with those treated with SA or No-tr (HA vs. SA, HA vs. No-tr, $p < 0.05$ in all doses). There were no significantly differences in intimal hyperplasia, subintimal fibrosis and vascularity among the three groups ($p > 0.05$).

Immunohistochemical assessments on location of HIF-1α, iNOS, and MMP3

Overexpressions of HIF-1α, iNOS, and MMP3 immunoreactivity were found within the synovial tissue in No-tr (figures 4A, 5A, 6A) and SA groups (figures 4B, 5B, 6B). At higher-power magnification, it is evident that these positive immunoreactivities were clearly localized in both nucleus and cytoplasm of arthritic synovium (figures 4a, 5a, 6a; 4b, 5b, 6b).

The primary cells exhibiting specific HIF-1α, iNOS, and MMP3 immunoreactivity were morphologically consistent with macrophages, mainly in inflammatory infiltrate and invasive pannus of the inflamed synovial joint. Synovial lining cells and some chondrocytes were also found to be HIF-1α, iNOS and MMP3 positive. After treatment with HA, HIF-1α, iNOS and MMP3 immunoreactivity were reduced (figures 4C, 5C, 6C) concurrent with reduced immunoreactivities localized in both nucleus and cytoplasm of arthritic synovium at higher-power magnification (figures 4c, 5c, 6c).

Quantitative analysis on extents of HIF-1α, iNOS, and MMP3

After treatment, the significant differences on extents of HIF-1α, iNOS, and MMP3 immunoreactive expression were shown among HA, SA, and No-tr groups after treatment of 1D (HIF-1α: $p < 0.001$; iNOS: $p < 0.001$; MMP3: $p < 0.001$), 3D (HIF-1α: $p < 0.001$; iNOS: $p < 0.001$; MMP3: $p < 0.001$), and 3D6d (HIF-1α: $p < 0.001$; iNOS: $p < 0.001$; MMP3: $p < 0.001$). Significantly lower expressions of HIF-1α, iNOS, and MMP3 immunoreactivity existed after treatment of 1D in HA groups (HIF-1α: HA vs. SA, $p < 0.001$; HA vs. No-tr, $p < 0.001$ [figure 4D]; iNOS: HA vs. SA, $p < 0.001$; HA vs. No-tr, $p < 0.001$ [figure 5D]; MMP3: HA vs. SA, $p < 0.001$; HA vs. No-tr, $p < 0.001$ [figure 6D]). The analysis also showed there were no significant differences on HIF-1α, iNOS, and MMP3 immunoreactivity between SA and No-tr groups for 1D dosage (HIF-1α: SA vs. No-tr, $p = 0.14$; iNOS: $p = 0.45$;

MMP3: $p = 1.0$, [figures 4D, 5D, 6D]). Similar results were also found on HIF-1α, iNOS, and MMP3 immunoreactivity for treatments of 3D and 3D6d (figures 4D, 5D, and 6D).

Significant difference on extents of HIF-1α, iNOS, and MMP3 immunoreactive expression were shown among 1D, 3D, and 3D6d dosages in HA group (HIF-1α: $p < 0.001$; iNOS: $p = 0.004$; MMP3: $p < 0.001$), but not in both SA (HIF-1α: $p = 0.56$; iNOS: $p = 0.85$; MMP3: $p = 0.81$) and No-tr (HIF-1α: $p = 0.16$; iNOS: $p = 0.50$; MMP3: $p = 0.99$) groups. After 3D and 3D6d of HA treatment, the extents of HIF-1α and iNOS immunoreactive expression significantly reached maximum reduction compared with those of 1D treatment (HIF-1α: 3D vs. 1D, $p < 0.001$; 3D6d vs. 1D, $p = 0.03$ [figure 4D]; iNOS: 3D vs. 1D, $p = 0.01$; 3D6d vs. 1D, $p = 0.03$ [figure 5D]). However, no difference was exhibited between the 3D and 3D6d of HA treatments (HIF-1α: 3D vs. 3D6d, $p = 0.15$; iNOS: 3D vs. 3D6d, $p = 1.0$). For expression of MMP3 immunoreactivity, significant reduction was found after 3D treatment (3D vs. 1D, $p = 0.001$; 3D vs. 3D6d, $p < 0.001$ [figure 6D]). However, the expression of MMP3 immunoreactivity recovered after 3D6d treatment (3D6d vs. 1D, $p = 1.0$).

Association of pain withdrawal threshold with immunoreactivity results

A significant linear correlation was found between pain withdrawal threshold and immunoreactivity of HIF-1α, iNOS and MMP3 (Pearson correlation coefficients, $p < 0.05$,

Table 1). There were strong negative association of pain withdrawal threshold with HIF-1α, iNOS and MMP3 after 3D treatment and those with HIF-1α and MMP3 after 3D6d treatment ($0.75 < |\text{Pearson } \gamma| < 1$).

Discussion

The results of this study demonstrate that lymphocytic/plasmocytic infiltration in the synovium and accumulation of HIF-1α, iNOS, and MMP3 were suppressed after intra-articular administration of HA at the early phase of adjuvant-induced inflammation. The extent of HIF-1α, iNOS, and MMP3 immunoreactivity was consistent with the results of pain behavioral assessment, which demonstrated elevation of the mechanonociceptive threshold after administration of HA. These findings have never been reported by other researchers.

In this model, the early phases of adjuvant-induced arthritis seem to be characterized by acute cytokines-induced inflammation [25]. Due to infiltration of the injured tissues caused by immune cells and responses, swelling is a major sign during acute inflammation and it might also be considered an important parameter on evaluation the potential anti-inflammatory effects of compounds [26]. However, as shown in the results of our study, The levels of edematous swelling were not changed after HA treatment in acute inflammation at the early phase of adjuvant-induced arthritis, suggesting the weaker activity against edema of HA in acute inflammatory animal model. This result is consistent with the animal study with collagen-induced arthritis [27] and human study with OA [28]. The reason is probably due to HA inducing swelling adverse effects which primarily occurs in the HA injected site. Previous studies revealed that HA may act either as a primary irritant or an inflammatory

mediator to induce acute adverse events characterized by transient swelling of the injected joint in some patients [28, 30, 31]. The prevalence of adverse effects was noted in 47% of patients after HA supplementation and in 22% of patients treated with saline injections (32). In this study, the observation time of edema measurement started 3 h post-HA administration after HA administration when an adverse effect of a transient increase in swelling at the injection site occurred. Therefore, the further study with long-term observations of joint swelling after ceasing HA was needed for clarifying the effect of exogenous HA on resolving RA-induced joint edema.

It has been well-established by animal behavioral and human clinical studies that elastoviscous solutions of HA could have an analgesic effect when injected into arthritic joints and if appropriately applied to patients with acute arthritic pain [33]. There was significantly less bradykinin found in the crystal-induced arthritic joint after the injection of HA [34]. Electrophysiological studies also demonstrated that the rate of neural discharges of the nociceptive afferent fibers innervating the synovial tissue were significantly attenuated and reached a constant rate 2–3 h after injection [33, 35, 36]. Treatment of HA showed an analgesic effect after the onset of cartilage destruction and pain in a rabbit OA model [37]. Our behavioral study is the first report on the analgesic effect of HA at decreased mechanical allodynia in a rat RA model, which is also consistent with the findings of previous studies. The intra-articular injection of HA also resulted in elevation of mechanonociceptive threshold,

364 which was in accordance with those of data determined by immunohistochemistry in this
365 study.

366 HA has been demonstrated to possess a therapeutic effect on OA studied by many
367 researchers. Macroscopic and microscopic evaluations revealed that HA has
368 chondroprotective effects in a rabbit model of OA [38]. Our results showed that HA reduced
369 the pathohistological sign including the degree of infiltration of the synovial membrane by
370 plasma and lymphocytes in CIA animals which is consistent with findings from previous
371 study (39). The tendency for decreased cellular infiltration during early phase of arthritis
372 supports the assumption that HA provides a temporary protecting barrier over the cartilage,
373 and thereby protects it against CFA insults. HA has also been shown to significantly suppress
374 NO production and inhibit interleukin-1 beta (IL-1ß)-induced MMP3 production from OA
375 synovial tissue in vitro and in vivo [40-43]. As far as we know in English literature, few
376 studies regarding the role of HA on suppression of HIF-1α-mediated hypoxic and
377 inflammatory responses have been conducted in OA models. Due to less inflammation in OA
378 synovial tissue, there is minor HIF-1α expression in these tissues [5]. However, there is
379 relatively higher expression of HIF-1α immunohistochemistry in RA synovial tissues
380 compared with OA tissues due to the nature of the tissue being inflammatory and angiogenic
381 in RA [7]. Therefore, HIF-1α has the potential to serve as an anti-rheumatic drug activity

biomarker in the clinic and is expected to significantly affect/accelerate the clinical development of treatment for RA.

The possible important role of HIF-1α in RA has been extensively discussed [44, 45]. The presence of both hypoxia and inflammatory proteins in RA synovium, which both lead to HIF-1α stabilization and subsequent HIF-1 activation, seems to highlight the important role of HIF-1α [45]. Elevated synovial angiogenesis is a key event during the course of RA. The modulation and blockade of angiogenesis via drug interventions has been shown to contribute to therapeutic efficacy in rat models of arthritis [46]. HIF-1α probably has an essential involvement in the angiogenic process of synovium in RA by regulation of its target gene, vascular endothelial growth factor (VEGF) (44). Inhibition of HIF-1α protein expression and VEGF production by SMP-114, a disease-modifying anti-rheumatic drug (DMARD), has been shown of therapeutic benefit in RA [47]. Oral administration of the inhibitor of heat shock protein 90 (Hsp90) which has been shown to potently reduce HIF-1α-related signaling and VEGF production has also been found to decrease inflammation and cartilage damage *in vivo* models of RA [48]. Therefore, suppression of HIF-1α may be a key implication on the development of novel therapeutic strategies revolutionizing the treatment of RA. Results showed that HA suppressed the adjuvant-induced overexpression of iNOS and MMP3, which is consistent with findings from previous studies. Our study is the first to report that HA suppresses HIF-1α. This study revealed the reduction of accumulation of HIF-1α expression

in synovium of adjuvant-induced RA model after intra-articular HA administration. The suppressive effects on accumulation of inflammation-induced HIF-1α, iNOS, and MMP3 expressions in synovium may be involved in the therapeutic mechanism of HA intervention used in treatment of RA. Further molecular studies on expressions of VEGF will be needed for fully supporting the issue of anti-angiogenic effects of HA.

Conclusions

Suppression of HIF-1α may be one of the major targets of the therapeutic approach in RA. This study demonstrated that early intervention of HA is an effective protection against accumulation of inflammation-induced HIF-1α, iNOS, and MMP3, which might limit the erosive joint damage of arthritis. The findings suggest that modulation of HIF-1 α as a "master switch" may be used as a therapeutic target in the anti-inflammatory treatment of RA.

List of abbreviations

1D: 1 dose, 3D: three doses, 3D6d: follow-up at the 6th day after 3 doses, CaMKII: Ca^{2+}/calmodulin-dependent kinase II, CFA: complete Freund's adjuvant, DMARD: disease-modifying anti-rheumatic drugs, H&E: hematoxylin and eosin, HA: hyaluronan,

HIF-1α: hypoxia-inducible factor-1 alpha, Hsp90: heat shock protein 90, IL-1ß: interleukin-1 beta, iNOS: inducible nitric oxide synthase, MMP3: matrix metalloproteinase-3, MMPs: matrix metalloproteinases, NO: Nitric oxide, NOS: nitric oxide synthases, OA: osteoarthritis, RA: rheumatoid arthritis, SD: Sprague-Dawley, SPSS: Statistical Package for the Social Sciences, VEGF: vascular endothelial growth factor.

Competing interests: The authors have declared no conflicts of interest.

Authors' contributions

LWC conceived the study, and participated in data analysis, and drafted the manuscript. JW participated in the histopathology and scored the immunohistology. PLC participated in the establishment of animal model, immunohistology and animals' functional evaluations. YLH conceived the study, performed the statistical analysis, and drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Experimental design of the sequence of events for the entire course of the experiment. After the evaluations including measurements of paw edematous swelling and pain threshold, the animals were sacrificed for histology and immunohistochemistry. 1D: one dose; 3D: three doses; 3D6d: follow-up at the 6th day after 3 doses. CFA: complete Freund's adjuvant; HA: hyaluronan; No-tr: No treatment; SA: saline

Figure 2. Results of edema (A) and pain behavioral (B) assessments. Data were calculated before treatment at the conditions of pre- and post-CFA-induced arthritis, after treatment at conditions of one injection (1D), three injections (3D) and follow-up 6 d after 3D (3D6d) in treatment (hyaluronan injection, HA), placebo (saline administration, SA) and control (sham injection, No-tr) groups. Each bar represents the mean ± SD in body weight and mean ± SEM in paw circumference and withdrawal threshold. #: $p < 0.05$, Student's t-test for comparison of pre- and post-arthritic condition before treatment. *$p < 0.05$, Bonferroni post hoc test for comparison of difference between groups at dosages of 1D, 3D and 3D6d after treatment.

Figure 3. Histopathology of arthritis joints. Representative HE sections of the hind paws obtained from adjuvant-induced arthritic animals treated with intra-articular three injections for No-tr (A), SA (B), and HA (C). In rats without any treatment for No-tr group, wherein

cartilaginous tissue could not be clearly detected, bone damage was even greater and there was massive inflammatory cells infiltrated in synovium (a). Similar changes were observed in rats treated with SA. Cartilage erosion was more pronounced and the extensively expanded synovial pannus was more densely infiltrated with mononuclear cells (b). In rats treated with HA, the joints were much less inflamed, and lymphocyte accumulation (c) and cartilage damage decreased. There was no sign of bone destruction (cart = cartilage; syn = synovial tissue; see figure 2 for other definitions).

Figure 4. Representative immunohistochemical sections of HIF-1α immunoreactivity. Sections obtained from the arthritic synovium treated with intra-articular three injections of No-tr (A), SA (B), and HA (C) groups. At higher-power magnification, it is evident that these positive (brown staining) immunoreactivities were clearly localized in both nucleus and cytoplasm of arthritic synovium in the sections from No-tr (a) and SA (b) animals. Administration of HA (c) to adjuvant-induced rat produced a marked reduction in the immunostaining for HIF-1α quantitative analysis (D) of positive-labeled cells in synovium for HIF-1α immunohistochemistry at the early phase of inflammation of each group was presented in the average proportion of labeled neurons (mean ± SEM). * $p < 0.05$, showed significant differences between groups when either SA or No-tr is compared with HA group using Bonferroni post hoc test. Significant differences were found between HA vs SA groups

and HA vs No-tr groups. $^{\#}$ $p < 0.05$, showed significant differences between dosages tested by Bonferroni post hoc test (cart = cartilage; syn = synovial tissue; see figure 2 for other definitions).

Figure 5. Representative immunohistochemical sections of iNOS immunoreactivity. Sections obtained from the arthritic synovium treated with intra-articular three injections of No-tr (A), SA (B), and HA (C) groups. At higher-power magnification, it is evident that these positive (brown staining) immunoreactivities were clearly localized in both nucleus and cytoplasm of arthritic synovium in the sections from No-tr (a) and SA (b) animals. Administration of HA (c) to adjuvant-induced rat produced a marked reduction in the immunostaining for iNOS. Quantitative analysis (D) of positive-labeled cells in synovium for iNOS immunohistochemistry at the early phase of inflammation of each group was presented in the average proportion of labeled neurons (mean ± SEM). * $p < 0.05$, showed significant differences between groups when either SA or No-tr is compared with HA group using Bonferroni post hoc test. Significant differences were found between HA vs SA groups and HA vs No-tr groups. $^{\#}$ $p < 0.05$, showed significant differences between dosages tested by Bonferroni post hoc test (cart = cartilage; syn = synovial tissue; see figure 2 for other definitions).

Figure 6. Representative immunohistochemical sections of MMP3 immunoreactivity. Sections obtained from the arthritic synovium treated with intra-articular three injections of No-tr (A), SA (B), and HA (C) groups. At higher-power magnification, it is evident that these positive (brown staining) immunoreactivities were clearly localized in both nucleus and cytoplasm of arthritic synovium in the sections from No-tr (a) and SA (b) animals. Administration of HA (c) to adjuvant-induced rat produced a marked reduction in the immunostaining for iNOS. Quantitative analysis (D) of positive-labeled cells in synovium for MMP3 immunohistochemistry at the early phase of inflammation of each group was presented in the average proportion of labeled neurons (mean ± SEM). * $p < 0.05$, showed significant differences between groups when either SA or No-tr is compared with HA group using Bonferroni post hoc test. Significant differences were found between HA vs SA groups and HA vs No-tr groups. $^{\#}$ $p < 0.05$, showed significant differences between dosages tested by Bonferroni post hoc test (cart = cartilage; syn = synovial tissue; see figure 2 for other definitions).

672 Table 1. Association of pain withdrawal threshold with the
673 immunoreactivity results given as γ-values

	Pain withdrawal threshold		
	1D	3D	3D6d
HIF-1α	-0.378**	-0.848**	-0.869**
iNOS	-0.280*	-0.782**	-0.765**
MMP-3	-0.420**	-0.823**	-0.856**

674 Correlations were analyzed by Pearson correlation coefficients.

675 **: P<0.01; *:P<0.05.

Table 2. Results of histopathological scores of synovium for H&E sections in arthritic ankle joint sampled from HA, SA and No-tr groups.

Dose	Group	Intimal hyperplasia	Subintimal fibrosis	Lymphocytic infiltration	Vascularity	Aggregate score
1D	HA	2.45±0.11	2.60±0.11	1.50±0.11*#	2.05±0.11	7.80±0.26*#
	SA	2.60±0.11	2.60±0.11	2.50±0.11	2.10±0.12	9.10±0.31
	No-tr	2.65±0.11	2.65±0.10	2.95±0.05	2.20±0.12	9.75±0.24
	[a]*p* value among groups	*p*>0.05	*p*>0.05	*p*<0.001	*p*>0.05	*p*<0.001
3D	HA	2.50±0.11	2.70±0.11	1.40±0.13*#	2.20±0.09	8.05±0.31*#
	SA	2.80±0.09	2.70±0.10	2.55±0.11	2.15±0.11	9.55±0.28
	No-tr	2.80±0.09	2.70±0.11	2.85±0.08	2.20±0.14	9.95±0.32
	[a]*p* value among groups	*p*>0.05	*p*>0.05	*p*<0.001	*p*>0.05	*p*<0.001
3D6d	HA	2.50±0.11	2.50±0.11	1.40±0.11*#	2.15±0.11	7.85±0.25*#
	SA	2.70±0.11	2.60±0.10	2.77±0.10	2.20±0.14	9.6±0.36
	No-tr	2.70±0.11	2.70±0.11	2.85±0.08	2.40±0.11	10.05±0.33
	[a]*p* value among groups	*p*>0.05	*p*>0.05	*p*<0.001	*p*>0.05	*p*<0.001

Values are mean± SEM. [a]: tested with Kruskal–Wallis test. *: $p < 0.05$, showed statistically significant differences between HA and SA groups; [#]: $p < 0.05$, showed statistically significant differences between HA and No-tr groups; Mann-Whitney U-ranked tests were used for between-group comparisons.