



## Research article

# Inhibitory activity of gintonin on inflammation in human IL-1 $\beta$ -stimulated fibroblast-like synoviocytes and collagen-induced arthritis in mice



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## ABSTRACT

**Background:** Gintonin is a newly derived glycolipoprotein from the roots of ginseng. The purpose of this study is to investigate the anti-arthritic efficacy of Gintonin on various proteases and inflammatory mediators that have an important role in arthritis.

**Methods:** Fibroblast-like synoviocytes (FLS) were treated with Gintonin and stimulated with interleukin (IL)-1 $\beta$  1 hour later. The antioxidant effect of Gintonin was measured using MitoSOX and H<sub>2</sub>DCFDA experiments. The anti-arthritic efficacy of Gintonin was examined by analyzing the expression levels of inflammatory mediators using RT-PCR, western blot, and ELISA. The phosphorylation of mitogen-activated protein kinase (MAPK) pathways and translocation of nuclear factor kappa B (NF- $\kappa$ B)/p65 into the nucleus were also analyzed using western blot, ELISA, and immunocytochemistry. Collagen-induced arthritis (CIA) mice model was used. Mice were orally administered with Gintonin (25, 50, and 100 mg/kg) every 2 days for 45 days. The body weight, arthritis score, squeaking score, and paw volume were measured as the behavioral parameters. After sacrifice, H&E and safranin-O staining were performed for histological analysis.

**Results:** Gintonin significantly inhibited the expression of inflammatory intermediates. Gintonin prevented NF- $\kappa$ B/p65 from moving into the nucleus through the JNK and ERK MAPK phosphorylation in FLS cells. Moreover, Gintonin suppressed the symptoms of arthritis in the CIA mice model.

**Conclusion:** As a result, the antioxidant and anti-inflammatory effects of Gintonin were demonstrated, and ultimately the anti-arthritic effect was proved. Collectively, Gintonin has a great potential as a therapeutic agent for arthritis treatment.

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## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in the synovial membrane (synovitis), resulting in the destruction and deformation of cartilage [1]. Common symptoms of arthritis include pain, edema, stiffness of joints, and fatigue [2]. The cause of rheumatoid arthritis has not been clarified yet, but the onset of RA begins with the induction of an inflammatory immune response in the synovial membrane. Depending on the inflammatory response, the continuation of the

synovitis would then lead to the destruction and deformation of the joints [3].

Typical inflammatory cytokines causing inflammatory responses and tissue destruction in inflammatory diseases such as arthritis include iNOS, TNF- $\alpha$ , IL-6, COX-2, and IL-1 $\beta$  [4]. iNOS is responsible for the generation of NO, and TNF- $\alpha$  aids in the progress of inflammation by inducing angiogenesis [5], and stimulates the generation of matrix metalloproteinases (MMPs) which are enzymes that can break down the components of extracellular matrix (ECM) in the articulated cartilage [6]. IL-1 $\beta$ , a representative inflammatory cytokine, plays an important role in cartilage decomposition. COX-2 amplifies the progression of local inflammation by increasing the expression of IL-1 $\beta$  and leads to the production of PGE<sub>2</sub> [7]. IL-6 increases the number of inflammatory cells in the

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synovial membrane and works with IL-1 $\beta$  to increase MMPs in chondrocytes [8–10].

Gintonin is a bioactive substance derived from the roots of ginseng [11]. The main components of Gintonin consist of lysophosphatidic acid (LPA), ribonuclease-like storage protein, and ginseng major latex-like protein 151 (GLP151) [12]. LPA is a well-known phospholipid molecule that acts extracellularly. LPAs originate from the cell membrane produced from lysophosphatidylcholine by autotoxin, an LPA synthetic enzyme. LPA works in conjunction with the LPA receptor, which have six types (subtype 1–6) and are also G-protein coupled receptors (GPCRs) [13,14].

So far, Gintonin has been reported as an active agent on Alzheimer's and Parkinson's diseases in various neuropathological studies [15–17]. Moreover the inhibitory effect of Gintonin on inflammation through MAPK and NF- $\kappa$ B in RAW264.7 cells was reported [18]. However, no arthritis-related effects of Gintonin have been reported until now. Therefore, the purpose of this study is to investigate the anti-arthritic effect of Gintonin by using stimulated FLS cells, and an experimental animal model of arthritis.

## 2. Materials and methods

### 2.1. Reagents

Gintonin was supplied from the Ginsentology Research Laboratory of Konkuk University (Seoul, Korea). All reagents used in cell culture were supplied by WELGENE Inc. (Gyeongsan, Korea) and Gibco (Grand Island, NY, USA). Complete-and incomplete- Freund's adjuvant, and chick type II collagen were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Human IL-1 $\beta$  was supplied by Bio Vision Inc. (Milpitas, CA, USA). Primers (iNOS, TNF- $\alpha$ , IL-6, COX-2, G-CSF, IL-1 $\alpha$ , IL-8, IL-18, MMP-1, MMP-3, MMP-13, and GAPDH) were purchased from Cosmo Genetech Co. (Seoul, Korea). Antibodies for  $\beta$ -actin, phosphorylated- and total-forms of p38, JNK/SAPK, ERK1/2, IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$ , and NF- $\kappa$ B/p65 were bought from Cell Signaling Technology (Danvers, MA, USA) and iNOS, TNF- $\alpha$ , IL-6 and COX-2 were supplied by Santa Cruz Biotechnology (Dallas, TX, USA).

### 2.2. Cell cultures

FLS cells were supplied by Cell Applications, Inc. (San Diego, CA, USA). The FLS cells are primary fibroblast-like cells derived from the synovial tissue of a RA patient, a 57-year-old Caucasian male. FLS cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 10  $\mu$ g/ml streptomycin under a humidified 5% CO<sub>2</sub> atmosphere at 37 °C. FLS cells between 3rd and 5th passages were used and media was changed every day until confluency reached 80%.

### 2.3. Cell viability

Cell viability was measured using Quanti-Max™ WST-8 cell viability assay kit (BIOMAX Co., Seoul, Korea). Cells were seeded to  $2 \times 10^4$  per well in a 96-well plate. Various concentrations of Gintonin were then added and after 1 hour, treated with IL-1 $\beta$  (10 ng/ml) for 24 hours. After incubation, to measure cell viability 10  $\mu$ l WST-8 was added for 1 h using a microplate reader at 450nm (Molecular Devices, San Jose, CA, USA).

### 2.4. Measurement of nitric oxide (NO) and ROS levels

FLS cells ( $2.5 \times 10^5$  cells/well in a 24-well plate) were prepared by treating under the same conditions as cell viability assay. The accumulated NO in the supernatant was measured using Griess

reagent (Promega, Madison, WI, USA) in accordance with the protocol provided by Promega. The intracellular ROS levels were detected using 7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA, Sigma-Aldrich, St. Louis, MO, USA). In brief, the cells were stained H<sub>2</sub>DCFDA solution dissolved at 50  $\mu$ M in pre-warmed PBS for 30 minutes at 37 °C and measured at an extinction of 485 nm and emission of 530 nm using a fluorescent reader (BioTek Instruments, Winooski, VT, USA). Mitochondrial ROS production was measured with MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen, Waltham, MA, USA) using the same method in accordance to a previous report [19]. Briefly, MitoSOX working solution (5  $\mu$ M) dissolved in HBSS/Ca/Mg was added into each well and incubated for 10 minutes at 37 °C. The fluorescence was monitored with a microplate reader set to 510 nm excitation and 595 nm emission wavelengths. For image analysis, cells were seeded and stained in the same way on a coverslip. Images were acquired using a confocal fluorescence microscope (Olympus, Tokyo, Japan).

### 2.5. Total RNA isolation, RT-PCR, and qRT-PCR analysis

Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). After reverse transcription with PrimeScript™ (TaKaRa Bio Co., Shiga, Japan), RT-PCR was performed using rTaq Plus 5x PCR master mix solution (ELPIS Biotech Inc., Daejeon, Korea), and quantitative RT-PCR was conducted using 2X Real-Time PCR master Mix including SFCgreenRI (BioFACT, Daejeon, Korea). GAPDH was used as a reference, and the data were analyzed using the  $\Delta\Delta$ Ct method. The primer sequences used are listed in supplement.

### 2.6. Western blot

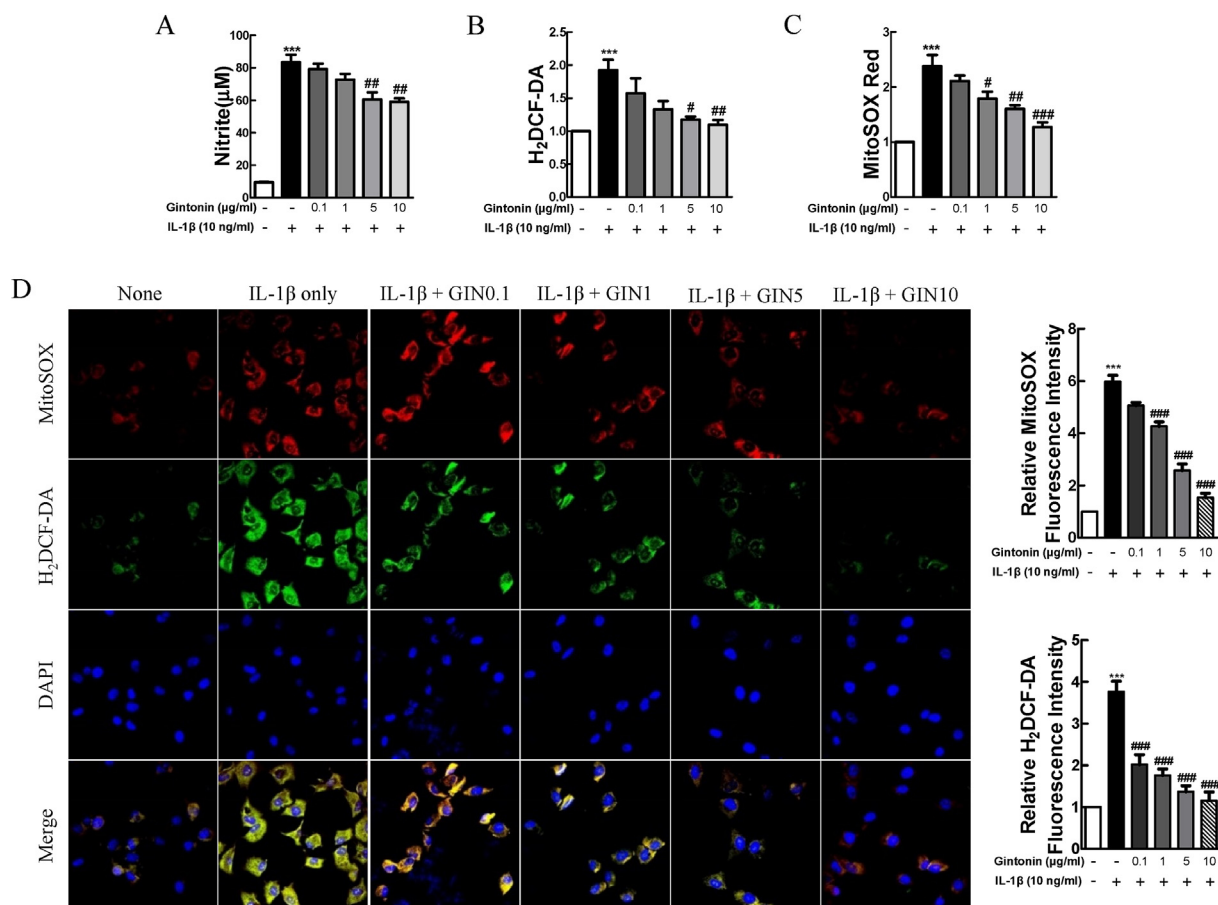
All cells were lysed using RIPA buffer (ELPIS Biotech Inc., Daejeon, Korea). The proteins were separated using 12% SDS-PAGE, transferred to a PVDF membrane, and were incubated in a chamber at 4 °C with the primary antibodies overnight, and with the secondary antibodies for 1 hour. The membranes were developed using the enhanced chemiluminescence (ECL) detection kits (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### 2.7. Enzyme-linked immunosorbent assay (ELISA)

The preparation process for the treatment of cells was the same as the NO measurement. The supernatant of the cells was measure with ELISA kits in accordance with the procedure provided by RayBiotech Inc. (Peachtree Corners, GA, USA). In case of measurement using serum obtained from mouse heart, it was measured with an ELISA kit purchased Elabscience biotechnology Inc. (Houston, TX, USA), and R&D Systems Inc. (Minneapolis, MN, USA) according to the protocol provided by each supplier.

### 2.8. Immunocytochemistry

Cells were cultured on a coated coverslip, pretreated with Gintonin, and stimulated with IL-1 $\beta$  1 hour later. After stimulation, the cells were incubated with 4% paraformaldehyde in PBS for 10 minutes and with 0.2% tritonX-100 in PBS for 5 minutes. After blocking in 5% horse serum and 2% BSA in PBS for 1 hour, cells were incubated with primary antibody specific for NF- $\kappa$ B/p65 (1:100) in 2% BSA in PBS at 4 °C overnight, and with Alexa Fluor 647-goat anti-rabbit immunoglobulin G secondary antibody (1:200) for 1 hour. The microscope slides were mounted using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA, USA) and image analysis was done using a confocal fluorescence microscope.



**Fig. 1.** Gintonin inhibited the production of NO and ROS in IL-1 $\beta$ -treated FLS cells. FLS cells were pretreated with Gintonin (0.1, 1, 5, 10  $\mu$ g/ml) for 1 h, followed by IL-1 $\beta$  (10 ng/ml) for 24 h. (A) Supernatant was used for nitrite measurements. (B) H<sub>2</sub>DCFDA for measurement of intracellular ROS and (C) MitoSOX red for detection of mitochondrial ROS production were used to stain cells. (D) Image analysis of ROS production. The cells were stained with MitoSOX (red), H<sub>2</sub>DCFDA (green), and DAPI (blue). The representative results from three independent experiments are shown. Quantification data are at the right panel. The data are expressed as means  $\pm$  S.E.M. (n = 3). \*\*\*p < 0.001 vs. untreated group (None), #p < 0.05 and ###p < 0.01, ####p < 0.001 vs. IL-1 $\beta$ -treated group without Gintonin treatment. None: non-treated cell, IL-1 $\beta$  only: IL-1 $\beta$  (10 ng/ml)-treated cell for 1 h, IL-1 $\beta$ +GIN0.1/GIN1/GIN5/GIN10: IL-1 $\beta$ (10 ng/ml) + Gintonin 0.1  $\mu$ g/ml/+ Gintonin 1  $\mu$ g/ml/+ Gintonin 5  $\mu$ g/ml/+ Gintonin 10  $\mu$ g/ml.

## 2.9. Collagen-induced polyarthritis in mice

Six-week-old male DBA/1J mice were randomly divided into five groups (n = 6); a non-treated normal group (NOR), a collagen-induced arthritis group (ART), collagen-induced arthritis and 25/50/100 mg/kg Gintonin-treated groups (ART + GIN25/50/100). On day 0, the mice were immunized by injecting 50  $\mu$ l of 2 mg/ml Chick type II collagen solution made with 2.5 ml of complete Freund's adjuvant and 2.5 ml of 0.05 N acetic acid subcutaneously at the base of the tail intradermally. On day 14, the mice were injected with a solution made by the same method but with 2.5 ml of incomplete Freund's adjuvant as a booster injection. Gintonin was dissolved in saline and starting from day 15, Gintonin (25, 50, and 100 mg/kg) was administered (P.O.) to treatment groups while normal and arthritis groups received only saline (P.O.) every two days until day 43. This animal experimental procedures were conducted according to the guidelines of the NIH and Ewha Womans University Institutional Animal Care and Use Committee.

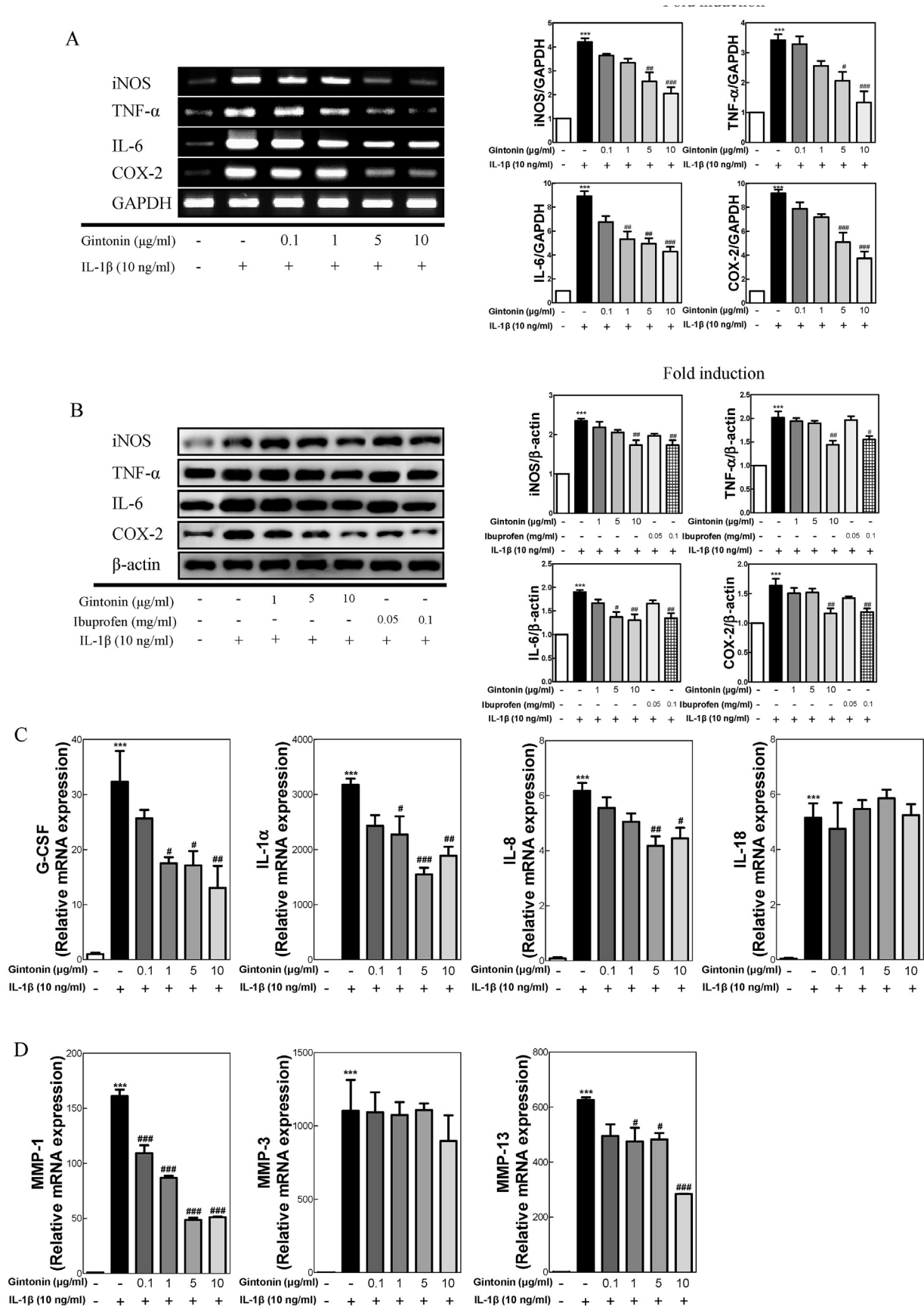
## 2.10. Assessment of arthritis symptoms

To measure the progress of collagen-induced arthritis in mice, four different parameters, body weight, paw volume, squeaking

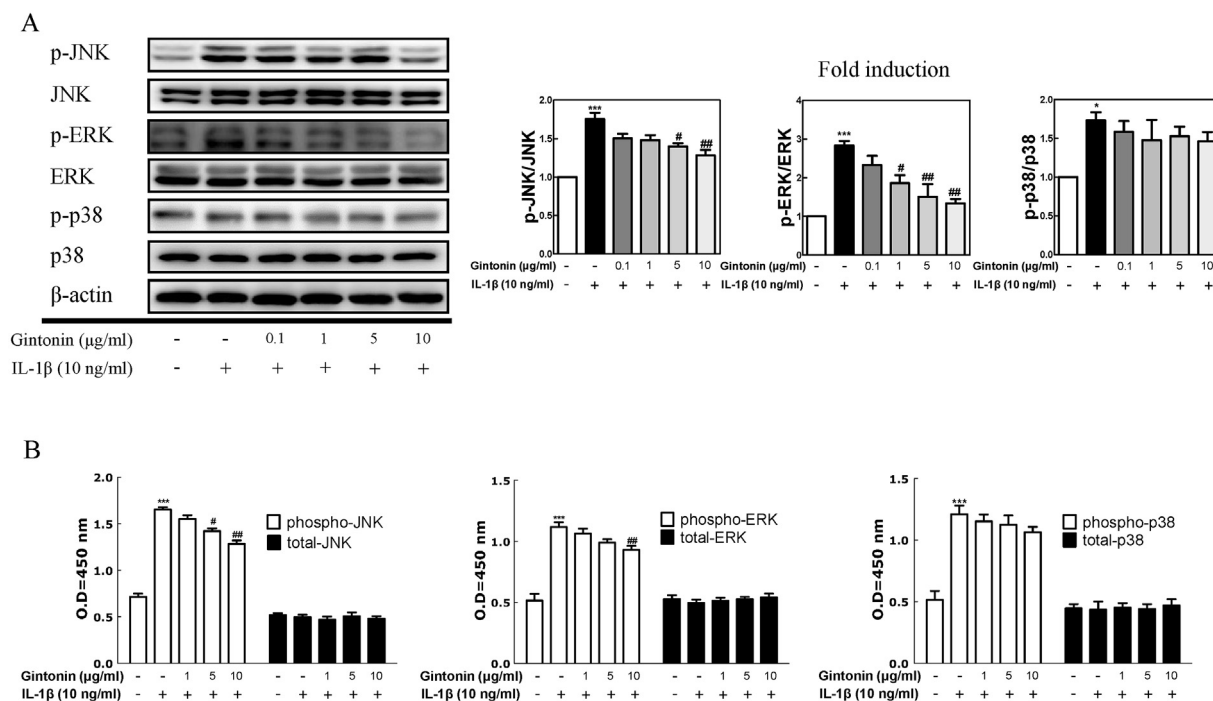
score, and arthritis score, were measured blindly every 2 days after the first immunization starting on day 0. The squeaking score was recorded as the average number of vocalizations when each hind leg was bent. The paw volume was assessed using a water digital plethysmometer (UGO-BASIL Biological Research Apparatus, Italy) [20]. As each hind limb was immersed into the electrolyte solution, the amount of liquid increased was recorded on a digital display. The arthritis index was conducted in accordance with the protocol provided by Hooke Laboratories, Inc. (Lawrence, MA, USA). The formation of pannus and synovitis were evaluated through H&E staining, and scored as described [21]. Erosion of cartilage was examined using Safranin-O staining and scored using Mankin's method as described [22,23].

## 2.11. Statistical analysis

All data are presented as mean  $\pm$  S.E.M. Data analyses were carried out using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical comparisons were identified using one-way ANOVA with Tukey's multiple comparison test, and two-way ANOVA with Bonferroni's post-hoc testing. P values < 0.05 were considered to indicate statistical significance.



**Fig. 2.** Gintonin downregulated mRNA levels and protein expression of pro-inflammatory mediators and MMPs in IL-1 $\beta$ -stimulated FLS cells. (A) RT-PCR, (B) Western blot and (C and D) qRT-PCR analysis using FLS cells pretreated with Gintonin for 1 h and stimulated later with IL-1 $\beta$  (10 ng/ml) for 6 hours. The GAPDH gene was used to normalize the mRNA levels of target genes. The representative results from three independent experiments are shown. Each quantification data is A and B at the right panel. The data are expressed as mean  $\pm$  S.E.M. (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. untreated group (None), #p < 0.05, ##p < 0.01, ###p < 0.001 vs. IL-1 $\beta$ -treated group without Gintonin treatment.



**Fig. 3.** Gintonin decreased MAPKs phosphorylation in IL-1 $\beta$ -treated FLS cells. (A) Western blot analysis and (B) ELISA analysis for MAPKs activities using FLS cells pretreated with Gintonin for 1 h and stimulated later with IL-1 $\beta$  (10 ng/ml) of 6 h. The representative results from three independent experiments are shown. Quantification data of the western blot are at the right panel. The data are expressed as mean  $\pm$  S.E.M. (n = 3). \*\*\*p < 0.001 vs. untreated group (None), #p < 0.05, ##p < 0.01 vs. IL-1 $\beta$ -treated group without Gintonin treatment.

### 3. Results

#### 3.1. Gintonin reduced the production of NO and ROS

Before starting *in vitro* experiments, Gintonin and IL-1 $\beta$  were confirmed to have no cytotoxicity in FLS cells. To determine whether Gintonin affects NO and ROS production, NO and ROS products were measured 24 hours after stimulating the cell with the IL-1 $\beta$  (10 ng/ml) following pretreatment with Gintonin for 1 hour. Gintonin significantly inhibited the production of NO (Fig. 1A) and ROS (Fig. 1B and C). In the case of ROS, both intracellular accumulation of ROS products (Fig. 1B) and superoxide production in the mitochondrial matrix (Fig. 1C) were reduced. ROS generation was not only measured using a plate reader, but also visualized by staining the cells (Fig. 1D). These results clearly demonstrated the inhibitory effect of Gintonin on NO and ROS products.

#### 3.2. Gintonin decreased the expression of inflammatory mediators

For further verification of anti-inflammatory effects of Gintonin, the mRNA levels as well as protein expression of representative inflammatory mediators, iNOS, TNF- $\alpha$ , IL-6, and COX-2, were identified through RT-PCR and western blot. First, RT-PCR analysis was used to determine whether Gintonin is involved in the transcription regulation of inflammatory factors in FLS cells. Stimulus of IL-1 $\beta$  increased the mRNA levels of each inflammatory mediator, and cells with Gintonin pretreatment decreased that levels in a dose-dependent manner (Fig. 2A). After confirming the mRNA levels, the protein expression showed a similar result (Fig. 2B). FLS cells stimulated with IL-1 $\beta$  caused an increase protein expression of iNOS, TNF- $\alpha$ , IL-6, and COX-2 and the pretreatment of Gintonin reduced the expression of iNOS, TNF- $\alpha$ , IL-6, and COX-2. Therefore these results suggested that Gintonin has an anti-inflammatory effect in FLS cells.

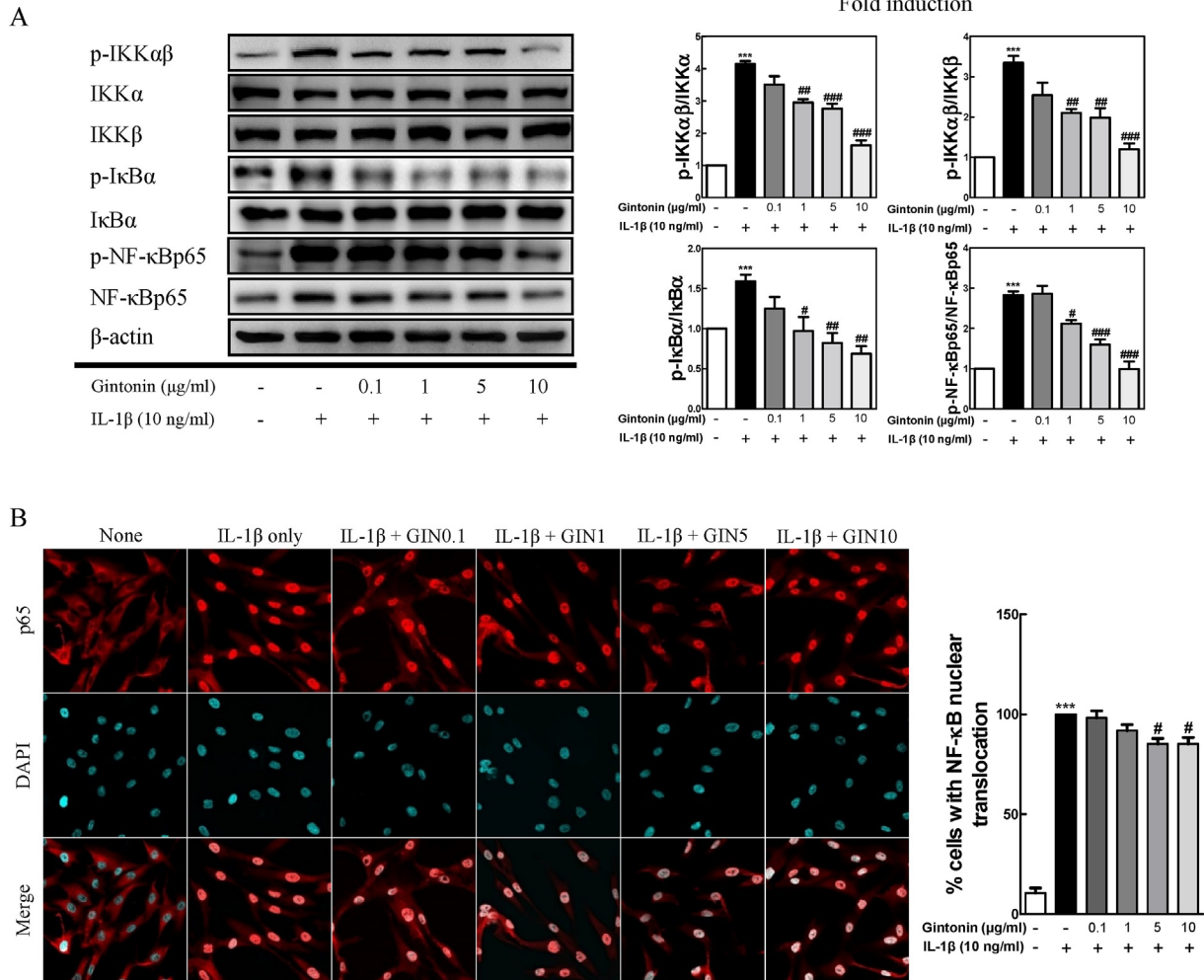
#### 3.3. Gintonin declined the expression of several protein targets and MMPs

In addition to the previously indicated inflammatory factors, cytokine array was performed to find additional targets that Gintonin influences. The detected four targets involved in inflammation or arthritis were further measured at the mRNA levels via qRT-PCR (Fig. 2C). The mRNA expression of G-CSF, IL-1 $\alpha$ , and IL-8 was decrease when Gintonin was pretreated compared to the IL-1 $\beta$  only stimulated group. To determine whether Gintonin affects the expression levels of MMPs, qRT-PCR analysis was performed (Fig. 2D). Among various MMPs, the collagenases MMP-1 and MMP-13 were chosen as interstitial collagen degradation markers, and the stromelysin MMP-3 as a non-collagen matrix protein degradation marker [24,25]. The mRNA levels of MMP-1 and MMP-13 were decreased by pretreatment of Gintonin in a dose-dependent manner, but the levels of MMP-3 remained unchanged.

#### 3.4. Gintonin regulated the MAPK signaling pathway

The MAPK pathways are involved in the upstream signaling of inflammatory processes. In order to determine the molecular mechanism behind the suppression of the aforementioned inflammatory mediators induced by Gintonin in FLS cells, this pathway was investigated using western blot and ELISA. Western blot analysis was conducted using the phosphorylated- and total-forms of antibodies against the extracellular signal regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 (Fig. 3A). Gintonin significantly reduced the phosphorylation of JNK and ERK MAPKs in IL-1 $\beta$ -stimulated FLS cells. However, Gintonin have no effect on the phosphorylation of p38 MAPK. As reflected in the western blot analysis, the ELISA results showed that Gintonin suppressed the phosphorylation of JNK and ERK MAPKs and had no effect in p38 MAPK phosphorylation (Fig. 3B).





**Fig. 4.** The effect of Gintonin on the NF- $\kappa$ B pathway in IL-1 $\beta$ -treated FLS cells. (A) Western blot analysis and (B) immunocytochemistry images for NF- $\kappa$ B pathway activities using FLS cells pretreated with Gintonin for 1 h and stimulated after with IL-1 $\beta$  (10 ng/ml) for 6 h. The cells were stained with p65 (red) and DAPI (blue). The representative results from three independent experiments are shown. Each quantification data is at the right panel. The data are expressed as mean  $\pm$  S.E.M. (n = 3). \*\*\*p < 0.001 vs. untreated group (None), #p < 0.05, ##p < 0.01, ###p < 0.001 vs. IL-1 $\beta$ -treated group without Gintonin treatment. None: non-treated cell, IL-1 $\beta$  only: IL-1 $\beta$  (10 ng/ml)-treated cell for 1 h, IL-1 $\beta$ +GIN0.1/GIN1/GIN5/GIN10: IL-1 $\beta$  (10 ng/ml) + Gintonin 0.1  $\mu$ g/ml/+ Gintonin 1  $\mu$ g/ml/+ Gintonin 5  $\mu$ g/ml/+ Gintonin 10  $\mu$ g/ml.

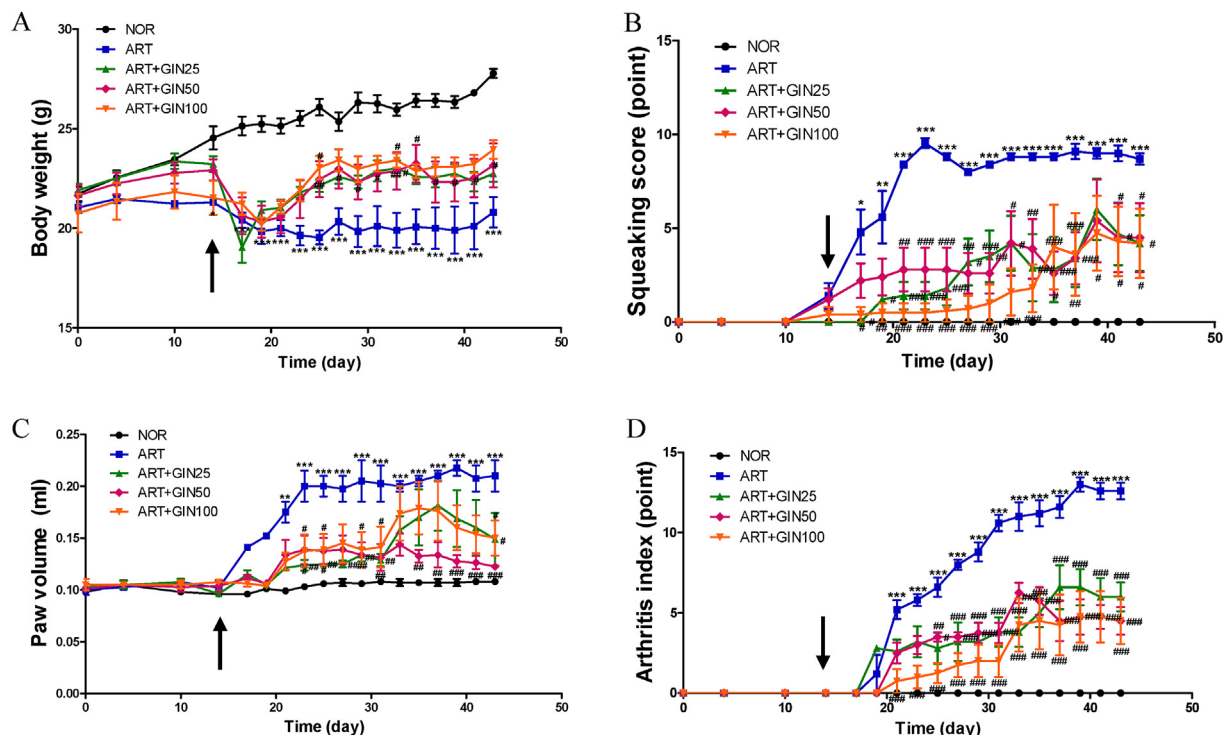
### 3.5. Gintonin regulated nuclear translocation of NF- $\kappa$ B/p65

NF- $\kappa$ B signaling pathway was analyzed using western blot (Fig. 4A) and immunocytochemistry (Fig. 4B). As expected, the results showed that the expression of phosphorylated IKK $\alpha$  $\beta$ , I $\kappa$ B $\alpha$ , and NF- $\kappa$ B/p65 was enhanced in IL-1 $\beta$ -stimulated FLS cells without Gintonin. On the other hand, the pretreatment of Gintonin down-regulated these activated protein expressions. The pretreatment of Gintonin at 10  $\mu$ g/ml inhibited the target signals the most. These results suggested that Gintonin blocked NF- $\kappa$ B/p65 from being transferred to the nucleus of IL-1 $\beta$ -stimulated FLS cells.

### 3.6. Gintonin relieved the arthritic symptoms in mice model

In order to further confirm Gintonin's efficacy in arthritis, an *in vivo* experiment was conducted using a polyarthritis animal model. As shown in Fig. 5A, the body weight of the mice showed decline immediately after the second immunization. A week after the second immunization (day 21), all three groups treated with Gintonin at 25, 50, and 100 mg/kg recovered slightly. The ART + GIN50/100 groups gained significant weight from day 25 and recovered substantially up to the last measurement day (day 43).

The ART + GIN25 group was also identified to have increasing weight but were not statistically significant. In the case of the squeaking score (Fig. 5B), ART group gradually increased the score after the secondary booster injection, and a high score was maintained after day 21. Other treatment groups also indicated an overall increase in squeaking scores after the second immunization, but the increase was significantly lower compared to the ART group. In regards to the paw volume (Fig. 5C), both hind limbs of ART group began to swell from day 17 and reached severe swelling up to day 23. All groups treated with Gintonin generally had a lower degree of swelling compared to ART group, and the rate of swelling was also slow. Arthritis index (Fig. 5D), which evaluates the comprehensive arthritis symptoms such as edema, inflammation, and erythema on the ankle joint of the arthritic mice, began to increase on day 19. For ART group, day 39 was maximal and was maintained afterwards. In all three groups administered with Gintonin, arthritis symptoms were weak and slow compared to ART group. As a result, Gintonin contributed to slower developing and lesser symptoms of arthritis compared to ART group in the collagen-induced arthritis (CIA) model. This suggests that Gintonin is effective against arthritis.



**Fig. 5.** Gintonin relieved the arthritic symptoms in the behavioral evaluation of the collagen-induced arthritis mouse model. (A) Body weight, (B) squeaking score (a value of 0 indicates no pain), (C) paw volume, and (D) arthritis index indicate the severity of arthritis in mice. The arrows indicate the date of the booster injection (day 14). From that date, Gintonin was administered once every two days until the day 43 when the experiment was completed. NOR: non-treated normal group, ART: collagen-induced arthritis group, ART + GIN25/GIN50/GIN100 = arthritis with Gintonin treatment at 25 mg/kg/50 mg/kg/100 mg/kg groups. All data are expressed as means  $\pm$  S.E.M. ( $n = 6$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. untreated group (NOR), # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. collagen-induced group without Gintonin treatment (two-way ANOVA followed by Bonferroni correction).

### 3.7. Gintonin indicated an anti-arthritis effect on the histopathological index

To observe the histopathological effects of Gintonin in the CIA model, knee joint tissue from each group of mice was collected and embedded in paraffin and sectioned. It was then stained with H&E and safranin-O. The results of H&E staining are shown in Fig. 6A along with typical foot images of the mice on the 43 day in the CIA model. The scores were evaluated mainly with a focus on the development of the pannus and cartilage-pannus junction and number of infiltrated immune cells. Gintonin generally showed a dose-dependent anti-arthritis effect compared to ART group. In the GIN100 treatment group, the development of the pannus decreased significantly enough to be visually identifiable. Thus, the anti-arthritis effects of Gintonin was identified in the CIA mice model.

Through safranin-O staining, which detects cartilage specifically, it is confirmed that Gintonin inhibits cartilage destruction (Fig. 6B). The upper images are knee joints stained with safranin-O, observed at  $\times 200$  magnifications. The yellow boxes in the upper image indicate the part that was enlarged to  $\times 400$  which can be seen in the lower image. ART group's knee cartilage was not clearly stained compared to NOR group, meaning that collagen treatment caused cartilage erosion. Gintonin's therapeutic increased the amount of stained cartilage.

### 3.8. Gintonin downregulated the expression of pro-inflammatory cytokines in the serum

To determine whether pretreatment with Gintonin inhibited inflammatory cytokines associated with arthritis, the sera of CIA

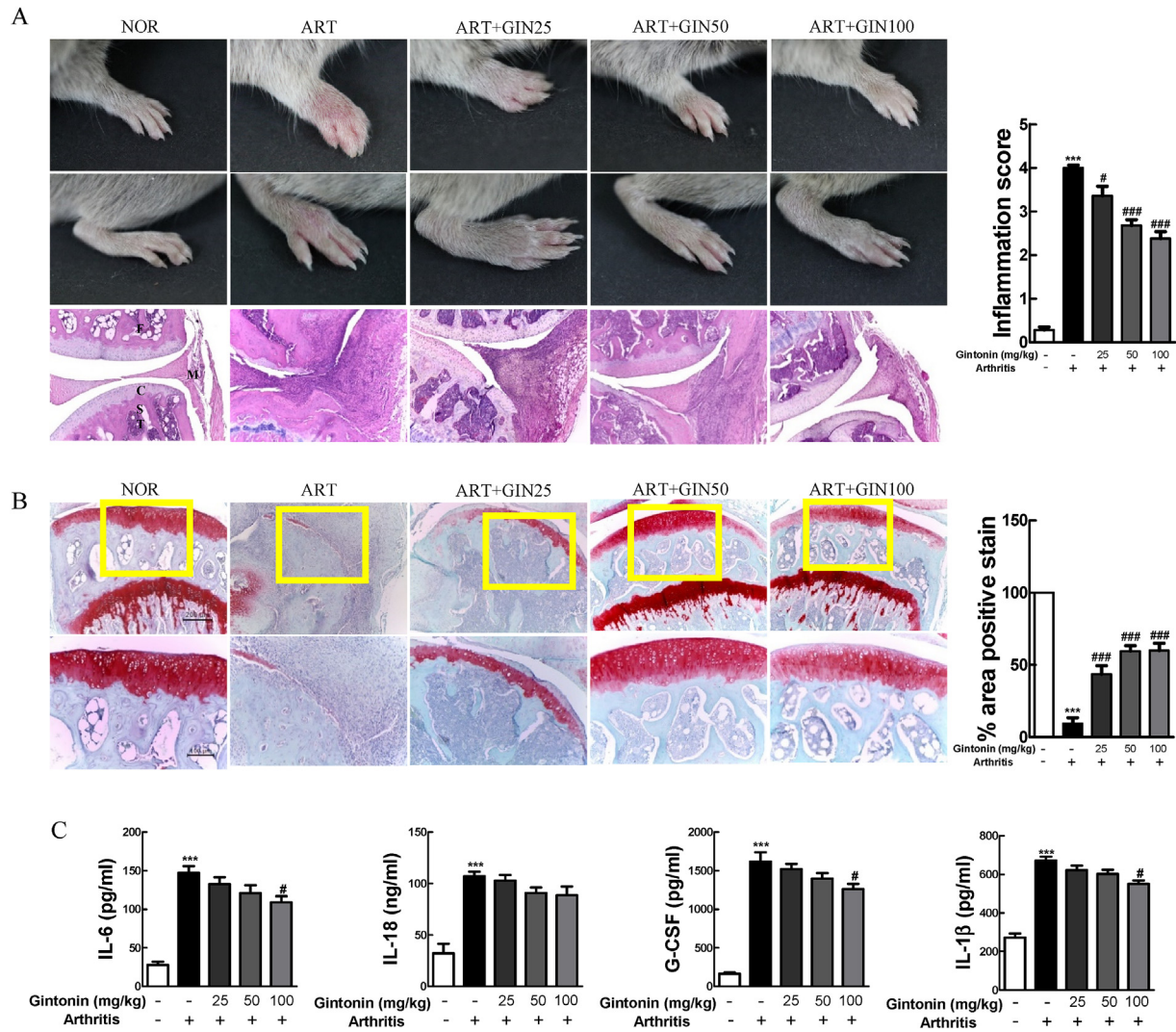
model mice were used to measure the levels of inflammation factors, IL-6, IL-18, and G-CSF (Fig. 6C). It was confirmed that the levels of each mediator were increased significantly in group ART, the arthritis group induced with collagen without any treatment with Gintonin. The inhibitory effect of Gintonin was not present in IL-18. However, Gintonin suppressed the levels of IL-6 and G-CSF significantly at a higher concentration of 100 mg/kg, and showed the anti-arthritis effect of Gintonin.

## 4. Discussion

There have been no reports about the effects of Gintonin on arthritis yet. Therefore, this is the first study that sequentially determined the bioactivity for antioxidant, anti-inflammatory, and consequentially, anti-arthritic effects of Gintonin.

At first, the antioxidant and anti-inflammatory action of Gintonin was verified using FLS cells. FLS cells stimulated with IL-1 $\beta$  increased the generation of NO and ROS which cause inflammatory reactions, cartilage decomposition, and apoptosis of chondrocytes in arthritis, along with an increase in the expression of inflammatory factors [26]. We targeted several common inflammatory intermediaries, iNOS, TNF- $\alpha$ , IL-6, COX-2, related to arthritis to measure how Gintonin affects the mRNA levels and protein expression in each target. The results showed that Gintonin decreased the generation of NO, ROS, and the mRNA levels of each target in IL-1 $\beta$ -stimulated FLS cells, and significantly inhibited the expression of inflammatory-related proteins. Thus, the antioxidant and anti-inflammatory effects of Gintonin have been demonstrated.

Four arthritis-related factors were found through a cytokine array, to explore whether the anti-arthritis effect of Gintonin occurs



**Fig. 6.** Gintonin showed an anti-arthritis effect in CIA model in mice. (A) Front paw, hind paw on day 43, and histological images of paraffin sections made from the knee of the mice stained with H&E ( $\times 100$ ). The extent of inflammation in the knee joint was graded between 0 and 4; 0 = normal (no infiltrate), 1 = minimal inflammation, 2 = mild inflammation, 3 = moderate inflammation, and 4 = severe inflammation. (B) Safranin-O staining images ( $\times 200$ ). The small yellow boxes indicate the photos below ( $\times 400$ ). The width of the stained cartilage was compared relative to the normal group. (C) ELISA experiments measuring the levels of IL-6, IL-18, and G-CSF in serum obtained from CIA mice. NOR: non-treated normal group, ART: collagen-induced arthritis group, ART + GIN25/GIN50/GIN100 = arthritis with Gintonin treatment at 25 mg/kg/50 mg/kg/100 mg/kg groups. C: cartilage, F: femur, M: meniscus, S: subchondral done, T: tibia. The representative results from three independent experiments are shown. All data are expressed as means  $\pm$  S.E.M. ( $n = 3$ ). \*\*\* $p < 0.001$  vs. untreated group (NOR), # $p < 0.05$ , ### $p < 0.001$  vs. carrageenan/kaolin-induced group without Gintonin treatment (ART) (one-way ANOVA followed by Tukey's multiple comparison test).

through other inflammatory mediators. The mRNA levels of these four targets were evaluated using qRT-PCR. Results showed that Gintonin decreased the expression of G-CSF, IL-1 $\alpha$ , and IL-8 although it did not affect the expression of IL-18.

In order to verify the effects of Gintonin in the suppression of MMP generation, the mRNA expression levels were measured by targeting MMP-1 and MMP-13, which are known to play an important role in ECM decomposition, and MMP-3, which is involved in non-collagen matrix degradation, through qRT-PCR [24]. As a result, Gintonin had a suppressive effect on the expression of MMP-1 and MMP-13.

In this process, we have found that treatment with Gintonin passes through the MAPK signaling pathway, which is mainly involved in direct cell reactions to various kinds of screening stimuli, including pro-inflammatory cytokines. In IL-1 $\beta$ -stimulated FLS cells, Gintonin inhibited the JNK and ERK1/2 MAPK signaling pathways, which were measured using Western blot and ELISA. In

addition, suppression of the downstream signaling on the NF- $\kappa$ B signaling pathway was revealed through Western blot and immunocytochemistry. Thus, the inhibition of MAPKs suggests that Gintonin blocked the nuclear translocation of NF- $\kappa$ B.

To further verify the anti-arthritis effect of Gintonin, we then carried out the animal experiment using an arthritis mouse model induced by collagen. The collagen-induced arthritis (CIA) mouse model is one of the most commonly used autoimmune models of rheumatoid arthritis. Like rheumatoid arthritis, erosion of cartilage and bone is a characteristic of CIA [27]. We used the following indicators, body weight, squeaking score, arthritis index, and paw volume to determine the severity of arthritis. The results showed that the pain was slowed down and alleviated by Gintonin. To further support the results of these behavioral experiments, in addition to observing the pannus with H&E staining, changes in the thickness of cartilage were observed using safranin-O staining. The anti-arthritis effect of Gintonin in the histological analysis was



consistent with the results of the behavioral experiments. The severity of pannus formation decreased and the erosion of cartilage was also reduced by Gintonin in a dose-dependent manner. Studies using the CIA model have reported that administration of IL-18 promotes the development of corrosive, inflammatory arthritis and IL-18 plays a pro-inflammatory role in rheumatoid arthritis [28]. Another interesting target, G-CSF, was also reported to be a potential treatment target for rheumatoid arthritis [29,30]. Therefore, we measured the expression of IL-6, IL-18, and G-CSF in the serum. As a result, Gintonin decreased the expression of IL-6 and G-CSF, but did not modulate IL-18 levels. Although the reduction effect of IL-18 did not appear, it fully supported the results of the CIA model's behavioral tests and histological analysis. With these, the anti-arthritis effect of Gintonin has been demonstrated in the animal experiment using CIA model.

Previous studies have shown that Gintonin induces a temporary increase in  $Ca^{2+}$  through the binding of LPA receptor (LPAR) 1-6 in oocytes [13,31]. In addition, it was shown that the activity of Gintonin-mediated LPAR increased neuronal excitability and slightly depolarized the resting membrane potential of hippocampal pyramidal neurons [32]. One of the possible action of Gintonin in arthritis would be mediated by activating of the LPA receptor.

In this study, we demonstrated the antioxidant and anti-inflammatory effects of Gintonin and ultimately the effect of anti-arthritis. It strongly suggests that such activation of Gintonin works through the LPAR. Collectively, it can be concluded that Gintonin has a great potential as a therapeutic agent for arthritis treatment.

### Conflicts of interest

The authors report no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2020.12.001>.

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