Brazilian Journal of Medical and Biological Research (2019) 52(12): e8525retraction, http://dx.doi.org/10.1590/1414-431X20198525retraction
ISSN 1414-431X Retraction

Retraction notice for: "Ginsenoside Rd inhibits IL-1β-induced inflammation and degradation of intervertebral disc chondrocytes by increasing IL1RAP ubiquitination" [Braz J Med Biol Res (2019) 52(9): e8525]

Wei-Jia Zhang[1], Ying Liu[1], Jie-Shu Wei[1], and Ya-Li Wu[2]

[1]School of Pharmacy, Xinhua College of Sun Yat-Sen University, Tianhe District, Guangzhou City, Guangzhou, China
[2]School of Rehabilitation Medicine, Xinhua College of Sun Yat-sen University, Tianhe District, Guangzhou City, Guangzhou, China

Retraction for: Braz J Med Biol Res | doi: 10.1590/1414-431x20198525 | PMCID: PMC6694592 | PMID: 31411316

The authors would like to retract the article ''Ginsenoside Rd inhibits IL-1β-induced inflammation and degradation of intervertebral disc chondrocytes by increasing IL1RAP ubiquitination'' that was published in volume 52 no. 9 (2019) (Epub Aug 12, 2019) in the Brazilian Journal of Medical and Biological Research <http://dx.doi.org/10.1590/1414-431x20198525> PMCID: PMC6694592 | PMID: 31411316.

The Corresponding author Ya-Li Wu states that "there is a conflict between authors concerning the publication of Figure 1 Aa and Ab, which had already been published in a Chinese Journal". Therefore, this article is being retracted and all authors will be prohibited to publish in the Brazilian Journal of Medical and Biological Research in the future.

Brazilian Journal of Medical and Biological Research (2019) 52(9): e8525, http://dx.doi.org/10.1590/1414-431X20198525
ISSN 1414-431X Research Article

Ginsenoside Rd inhibits IL-1β-induced inflammation and degradation of intervertebral disc chondrocytes by increasing IL1RAP ubiquitination

Wei-Jia Zhang[1], Ying Liu[1], Jie-Shu Wei[1] and Ya-Li Wu[2]

[1]School of Pharmacy, Xinhua College of Sun Yat-Sen University, Tianhe District, Guangzhou City, Guangzhou, China
[2]School of Rehabilitation Medicine, Xinhua College of Sun Yat-sen University, Tianhe District, Guangzhou City, Guangzhou, China

Abstract

Many compounds of ginsenosides show anti-inflammatory properties. However, their anti-inflammatory effects in intervertebral chondrocytes in the presence of inflammatory factors have never been shown. Increased levels of pro-inflammatory cytokines are generally associated with the degradation and death of chondrocytes; therefore, finding an effective and nontoxic substance that attenuates the inflammation is worthwhile. In this study, chondrocytes were isolated from the nucleus pulposus tissues, and the cells were treated with ginsenoside compounds and IL-1β, alone and in combination. Cell viability and death rate were assessed by CCK-8 and flow cytometry methods, respectively. PCR, western blot, and immunoprecipitation assays were performed to determine the mRNA and protein expression, and the interactions between proteins, respectively. Monomeric component of ginsenoside Rd had no toxicity at the tested range of concentrations. Furthermore, Rd suppressed the inflammatory response of chondrocytes to interleukin (IL)-1β by suppressing the increase in IL-1β, tumor necrosis factor (TNF)-α, IL-6, COX-2, and inducible nitric oxide synthase (iNOS) expression, and retarding IL-1β-induced degradation of chondrocytes by improving cell proliferation characteristics and expression of aggrecan and COL2A1. These protective effects of Rd were associated with ubiquitination of IL-1 receptor accessory protein (IL1RAP), blocking the stimulation of IL-1β to NF-κB. Bioinformatics analysis showed that NEDD4, CBLB, CBLC, and ITCH most likely target IL1RAP. Rd increased intracellular ITCH level and the amount of ITCH attached to IL1RAP. Thus, IL1RAP ubiquitination promoted by Rd is likely to occur by up-regulation of ITCH. In summary, Rd inhibits IL-1β-induced inflammation and degradation of intervertebral disc chondrocytes by increasing IL1RAP ubiquitination.

Key words: Ginsenoside Rd; IL-1β; Inflammation; Degradation; Intervertebral disc chondrocytes; IL1RAP ubiquitination

Introduction

Degeneration of the intervertebral disc (IVD) is one of the main factors in the development of low back pain (LBP). LBP is a highly debilitating symptom associated with disability, activity limitation, and loss of productivity, and affects up to 80% of the world population (1). Although current conservative and surgical therapies are relatively effective in relieving pain temporarily, there is no therapy that can effectively stop or reverse the degenerative process. The process of IVD degeneration is driven by various stimuli, including genetic risk, mechanical trauma, injuries, smoking, obesity, and ageing. However, it is not completely clear how these factors induce aberrant molecular behavior and cell biology leading to IVD pathogenesis (1,2).

Degenerative disc disease is associated with increased levels of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), IL-6, and IL-8 in the microenvironment of IVD. These cytokines can be produced by native nucleus pulposus (NP) and annulus fibrosus cells as well as by infiltrating inflammatory cells. Upon IVD injury, macrophages and mast cells release IL-1β and TNF-α in the IVD, and induce native IVD cells to further produce pro-inflammatory cytokines (1,3). These pro-inflammatory cytokines disturb the anabolic and catabolic balance in IVD cells, accelerate cell senescence and death, and shift extracellular matrix composition, resulting in various changes in the tissue architecture and functions of IVD (1,3). These changes are associated with other pathological changes, such as neurovascular ingrowth into the IVD, sensitization of nervous system (i.e., upregulation of pain-related neuropeptides in the dorsal root ganglia), impingement of adjacent nerve roots, and increased growth of surrounding spinal muscles, which consequently cause LBP.

Correspondence: Ya-Li Wu: <wyl2019110@163.com>

Received January 22, 2019 | *Accepted May 29, 2019*

Panax ginseng is a widely used medicinal herb, of which ginsenosides are the major bioactive components. Ginsenosides are a group of saponins with a dammarane triterpenoid structure, mainly including Rb1, Rb3, Rd, Rg1, and Ro compounds. Many studies have demonstrated the medicinal value of ginseng ginsenoside compounds in the treatment of osteoarthritis, liver diseases, colitis, and tumors (4–7). Notably, the anti-inflammatory effect of ginsenosides plays a key role in the treatment of osteoarthritis. Intragastrical treatment with ginsenoside Rg5 or intra-articular injection of Rb1 reduced the levels of IL-1β, TNF-α, nitric oxide, and inducible nitric oxide synthetase (iNOS) in an osteoarthritis rat model (8,9). Moreover, ginsenoside Rb1 inhibits the impaired action of IL-1β on human articular chondrocytes by decreasing the levels of prostaglandin E2, NO^{2-}, matrix metalloproteinase-13 (MMP-13), cyclooxygenase-2 (COX-2), iNOS, caspase-3, and PARP, and increasing aggrecan and COL2A1 gene expression levels (10). Therefore, Rb1 attenuates IL-1β-induced cell inflammation and apoptosis. Ginsenoside Rg1 has also been reported to inhibit IL-1β-induced gene and protein expressions of MMP-13, COX-2, and PGE2 in human articular chondrocytes and to prevent type II collagen and aggrecan degradation in a dose-dependent manner (4). While the anti-inflammatory effect of ginsenosides has been identified in articular chondrocytes, whether a similar anti-inflammatory effect is shown in IVD cartilage remains unclear. The present study aimed to identify the immunomodulatory effects of ginsenosides in IVD chondrocytes and elucidate the underlying mechanism.

Material and Methods

Sample collection for primary culture

Patients (four males and four females, mean age: 56 years, range: 43–72) with LBP caused by disc herniation in the lumbar region were enrolled in this study. All participants provided written informed consent. The present study was approved by the local ethics committee of Guangdong Second Hospital of Traditional Chinese Medicine (China). These patients underwent lumbar discectomy by the technology of minimally invasive epiduroscopy in Guangdong Second Hospital of Traditional Chinese Medicine during January 2018. The NP tissues extracted during the surgery were used to isolate chondrocytes for the *in vitro* study. Tissues were enzymatically digested using a mixture of 0.2% collagenase II (Sigma-Aldrich, USA) and 0.25% trypsin (Sigma-Aldrich) for 4–6 h at 37°C. Isolated primary cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM/F12) (Gibco, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1% antibiotics-antimycotics (A/A) (Gibco) in a humidified 37°C 5% CO_2 incubator.

Immunocytochemistry assays

Chondrocytes were cultured on coverslips in 6-well plates. Cells were fixed with 4% paraformaldehyde for 10 min at 37°C and with methanol at −20°C for 2 min. Normal goat serum (10%; Hyclone; GE Healthcare Life Sciences, USA) was added to cells for 30 min to block nonspecific binding sites. The fixed cells were immunostained with primary antibodies targeting collagen II (dilution 1:800, Abcam, UK) overnight at 4°C and the Alexa Fluor 488-conjugated secondary antibody (1:500 dilution, catalog No. SP-9000; ZSGB-BIO, China) for 1 h at 37°C. Images were acquired with a high-resolution CoolSNAP™ CCD camera (Photometrics Inc., USA) under the control of a computer using Leica IM4000 software version 1.2 (Leica Microsystems Ltd., UK).

Toluidine blue O staining

Chondrocytes were stained with 0.04% w/v toluidine blue O (Sigma-Aldrich, T0394) in 0.1 mol/L sodium acetate (pH 4.0) for 10 min, rinsed, air-dried, and mounted.

Cell treatment

After two passages, chondrocytes were seeded in 96-well plates (0.4×10^5 cells/well), 12-well plates (1×10^5 cells/well), or 6-well plates (2×10^5 cells/well). The next day, the cells were serum-starved for 2 h and exposed to monomeric components of ginsenoside (Rb1, Rb3, Rd, Rg1, or Ro, purity 98.55%, batch number: MUST-16012503, Chengdu Institute of Biology, Chinese Academy of Sciences, China) or ginsenoside-free medium as a control for 12 h. Afterwards, the cells were cultured with IL-1β (Sigma-Aldrich) for 12 h.

Cell viability measurement

Cell viability was measured using CCK-8 (Sigma-Aldrich). Briefly, 0.5×10^4 cells were seeded in each 96-well plate for 24 h. Following seeding and treatments, CCK-8 reagents were added to each well at a final concentration of 10%. After incubating for 1 h, absorbance at 490 nm in each well was determined by a microplate reader (Boehringer Mannheim ES700, UK).

Flow cytometry method

After cell treatment, cells were stained using Annexin V-FITC/PI Apoptosis Detection kit I (Kaiji Biological Inc., China) according to the manufacturer's instructions. The rate of apoptosis was analyzed using a dual laser flow cytometer (Becton Dickinson, USA) and estimated using the ModFit LT software v. 1.0 (Verity Software House, USA).

RT-qPCR

RNA was extracted with TRIzol/chloroform (15596-018, Invitrogen, USA) according to the manufacturer's instructions. cDNA (1 μg) was reverse transcribed from RNA

Table 1. Primers used in PCR assay.

Name	Sequence (5′–3′)	Tm (°C)	Amplicon Size (bp)
IL-1β			
Forward	AGCTACGAATCTCCGACCAC	61	186
Reverse	CGTTATCCCATGTGTCGAAGAA	61	186
TNF-α			
Forward	CCTCTCTCTAATCAGCCCTCTG	60	220
Reverse	GAGGACCTGGGAGTAGATGAG	60	220
IL-6			
Forward	ACTCACCTCTTCAGAACGAATTG	61	149
Reverse	CCATCTTTGGAAGGTTCAGGTTG	61	[illegible]
COX-2			
Forward	GAGAGATGTATCCTCCCACAGTCA	60	117
Reverse	GACCAGGCACCAGACCAAAG	60	117
iNOS			
Forward	TTCAGTATCACAACCTCAGCAAG	60	207
Reverse	TGGACCTGCAAGTTAAAATCCC	60	207
Aggrecan			
Forward	ACTCTGGGTTTTCGTGACTCT	[illegible]	81
Reverse	ACACTCAGCGAGTTGTCATGG	61	81
Col2A1			
Forward	TGGACGCCATGAAGGTTT[illegible]CT	62	183
Reverse	TGGGAGCCAGATTGTCA[illegible]TC	62	183
MMP-3			
Forward	CTGGACTCCGAC[illegible]CTCT[illegible]GA	62	79
Reverse	CAGGAAAGGTTC[illegible]A[illegible]TGAC[illegible]	62	79
MMP-13			
Forward	ACTGAGAG[illegible]CCGAGA[illegible]ATG	61	103
Reverse	GAACCCCGCA[illegible]TTGGCTT	61	103
ITCH			
Forward	TG[illegible]GATGGCTCCAGATCCAA	60	94
Reverse	GACT[illegible]CCTAT[illegible]TCACCAGCTC	60	94

using a reverse transcription kit (Applied Biosystems, USA) and mixed with the primers (Table 1) and Fast Universal Master Mix (Applied Biosystems). Gene expression was examined by RT-qPCR. Data were analyzed by the comparative $2^{-\Delta\Delta t}$ method, with GAPDH as housekeeping gene. Results are reported as gene expression relative to control (fold-change).

ELISA

Levels of IL-1β, TNF-α, IL-6, and prostaglandin E2 (PGE_2) in culture medium were measured using ELISA kits (Sigma-Aldrich) according to the manufacturer's protocol. To avoid the interference from exogenous IL-1β, cells were washed three times and cultured in new fresh medium after treatment with IL-1β for 12 h. The cell medium after 24-h cultivation was collected for the ELISA measurement. Each sample was assessed by comparing to a standard curve. Absorbance was measured at 450 nm using the microplate reader (Boehringer Mannheim ES700).

Nitric oxide (NO) measurement

NO in culture medium was measured using the Griess reaction. Briefly, 100 μL cell culture medium was mixed with 100 μL Griess reagent (equal volumes of 1% (weight/vol) sulfanilamide in 5% (vol/vol) phosphoric acid and 0.1% (weight/vol) naphtylethylenediamine-HCl), incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured using the microplate reader (Boehringer Mannheim ES700).

Western blotting

Cells were lysed and mixed with Laemmli buffer (Sigma-Aldrich). The lysates were boiled (96°C, 5 min) and loaded onto 4–20% SDS-polyacrylamide gels. Proteins were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. Following transfer, the membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween (TBS-T) for 1 h at room temperature, and primary antibodies against p65 (1:1000; ab16502, Abcam), phospho (p)-p65 (1:1000; ab76302, Abcam),

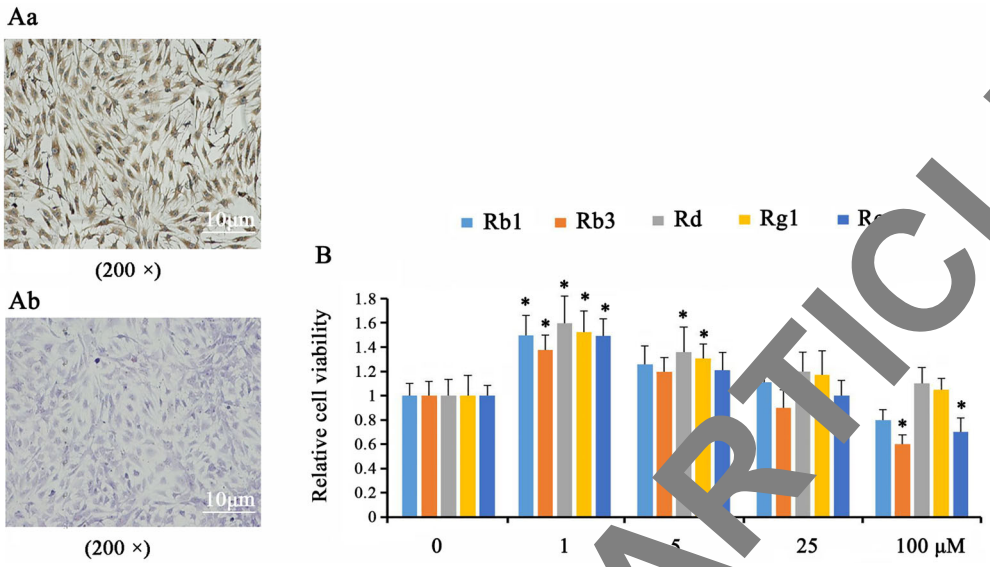

Figure 1. The effect of monomeric components of ginsenoside on the viability of intervertebral disc nucleus pulposus (NP) chondrocytes. Chondrocytes isolated from intervertebral disc NP underwent immunocytochemistry (**Aa**) and toluidine blue-staining assays (**Ab**) (magnification 200×, bars 10 μm). **B**, The effect of monomeric components of ginsenoside (Rb1, Rb3, Rd, Rg1, and Ro) on the viability of intervertebral disc NP chondrocytes was assessed by CCK-8. Data are reported as means ± SD. *$P<0.05$ *vs* control (ANOVA).

IL-1 receptor type I (IL-1RI) (1:500; ab106278, Abcam), IL-1 receptor accessory protein (IL1RAP) (1:1000; ab5110, Abcam), ITCH (1:500; ab220637, Abcam), and GAPDH (1:1000; ab181602, Abcam) were added overnight at 4°C under gentle shaking. The next day, membranes were washed in 1% non-fat milk in TBS-T (3 × 10 min) and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Visualization was performed by the chemiluminescence kit West Dura (Thermo Scientific, USA) on a ChemiDoc imager (Bio-Rad). The obtained bands were quantified using ImageJ (NIH, USA) × 64 by normalizing to loading control. Resulting graphs show an average of three independent donors.

Immunoprecipitation assay

Cells were lysed with immunoprecipitation assay lysis buffer (RIPA, Sigma-Aldrich). Cell lysates with equal amounts of protein (500 μg) were incubated with nickel beads conjugated to anti-IL1RAP antibody (Abcam) for 3 h, followed by washing with IP buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.5% NP-40). Bound proteins were detected by western blotting using primary antibodies against ubiquitin and ITCH (Abcam) and HRP-conjugated secondary antibody.

Statistical analysis

Each experiment was repeated three times. Data are reported as means ± SD, and one-way ANOVA was used to compare the means of independent samples. Statistical analyses were performed with SPSS 16.0 (SPSS Inc., USA). $P<0.05$ was considered statistically significant.

Ginsenoside toxicity in intervertebral disc chondrocytes

As indicated by immunocytochemistry, more than 95% of the isolated cells were cartilage cells (Figure 1Aa). Moreover, in the toluidine blue-staining assay, the cartilage cells showed normal morphology, namely, polygonal or spindle-shape, and relatively good growth performance (Figure 1Ab). Therefore, the isolated cells were acceptable for an *in vitro* study of cartilage cells.

The present study initially assessed the toxicity of the major monomeric components of ginsenoside (Rb1, Rb3, Rd, Rg1, and Ro) in intervertebral disc NP chondrocytes. According to the data from MTT assays, the viability of NP chondrocytes was increased by all of these monomeric components of ginsenoside at the dose of 1 μM and by both Rd and Rg1 at the dose of 5 μM ($P<0.05$, Figure 1B). Both Rb3 and Ro at the dose of 100 μM decreased NP chondrocyte viability ($P<0.05$, Figure 1B). However, Rb1, Rd, and Rg1 at such dosage had no toxicity. Notably, NP chondrocytes had the highest tolerance to Rd. Thus, Rd was used for further study.

Rd suppressed inflammatory response of NP chondrocytes to IL-1β

Rd at dosages of both 1 μM and 5 μM improved NP chondrocyte viability. This study further investigated the effect of 1 μM and 5 μM Rd on the inflammatory response of NP chondrocytes to IL-1β. NP chondrocytes showed increased mRNA expression of IL-1β ($P<0.01$), TNF-α ($P<0.05$), IL-6 ($P<0.05$), COX-2 ($P<0.05$), and iNOS ($P<0.05$) in response to IL-1β (Figure 2A).

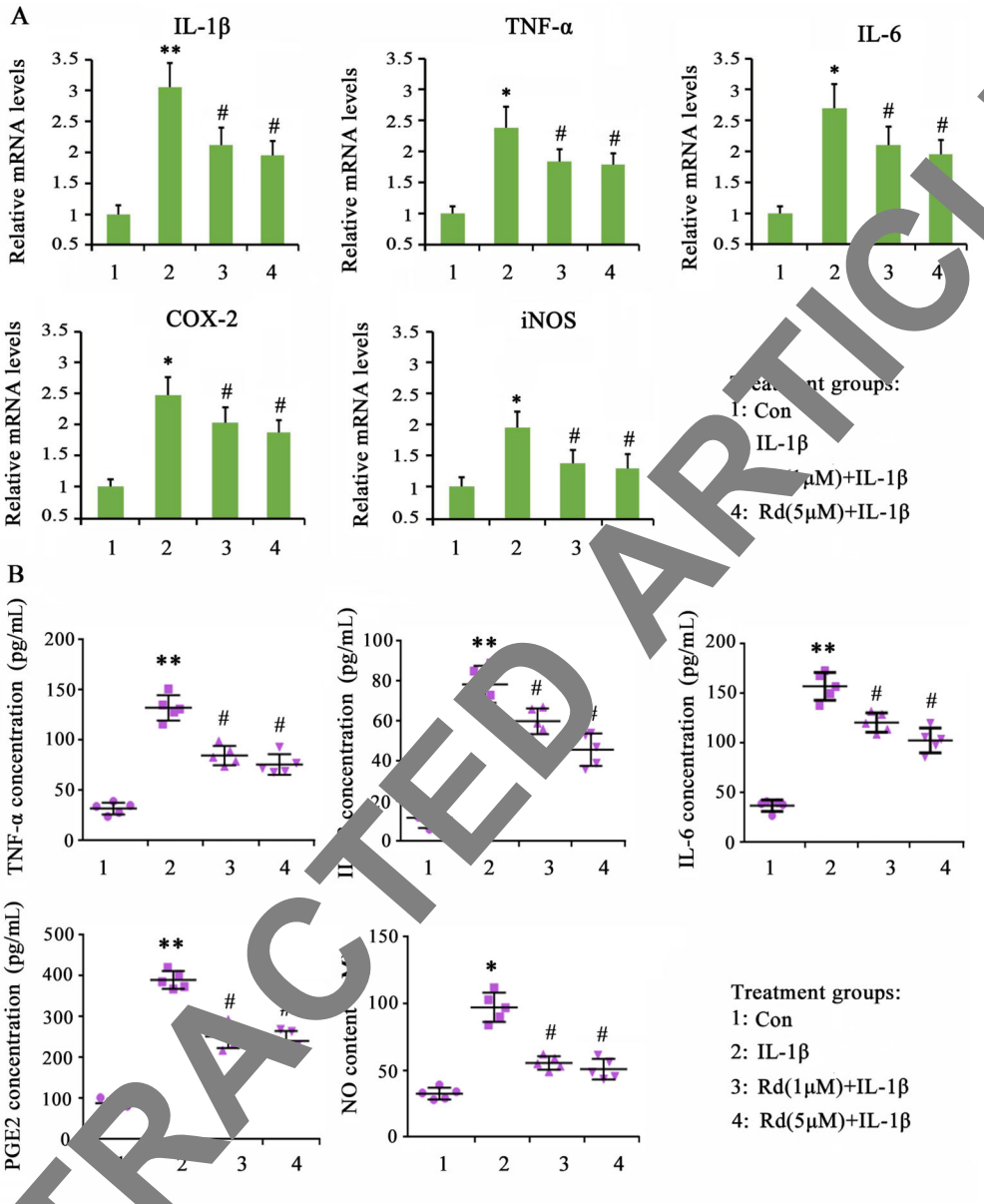

Figure 2. Ginsenoside Rd suppressed inflammatory response of nucleus pulposus (NP) chondrocytes to IL-1β. NP chondrocytes were treated with Rd prior to interleukin (IL)-1β. **A**, mRNA expression profiles in the cells were determined by PCR. **B**, Concentrations of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and PGE2 in the cell medium were assessed by ELISA assay. Nitric oxide (NO) content in the cell medium was evaluated using Griess agent. Data are reported as means ± SD. *P < 0.05 and **P < 0.01 *vs* control; #P < 0.05 *vs* IL-1β group (ANOVA).

However, pre-treatment with Rd (1 μM or 5 μM) mitigated the increase in these pro-inflammatory factors (P < 0.05). NO and PGE2 are the products of iNOS and COX-2, respectively. In line with the mRNA expression, the concentrations of IL-1β (P < 0.01), TNF-α (P < 0.01), IL-6 (P < 0.01), NO (P < 0.05), and PGE2 (P < 0.01) in culture medium were increased after IL-1β treatment (Figure 2B). However, pre-treatment with 1 and 5 μM Rd hindered the increase in these pro-inflammatory factors (P < 0.05)

Rd retarded IL-1β-induced degradation of NP chondrocytes

As indicated by CCK-8 assays, the viability of NP chondrocytes was impaired after the treatment with IL-1β

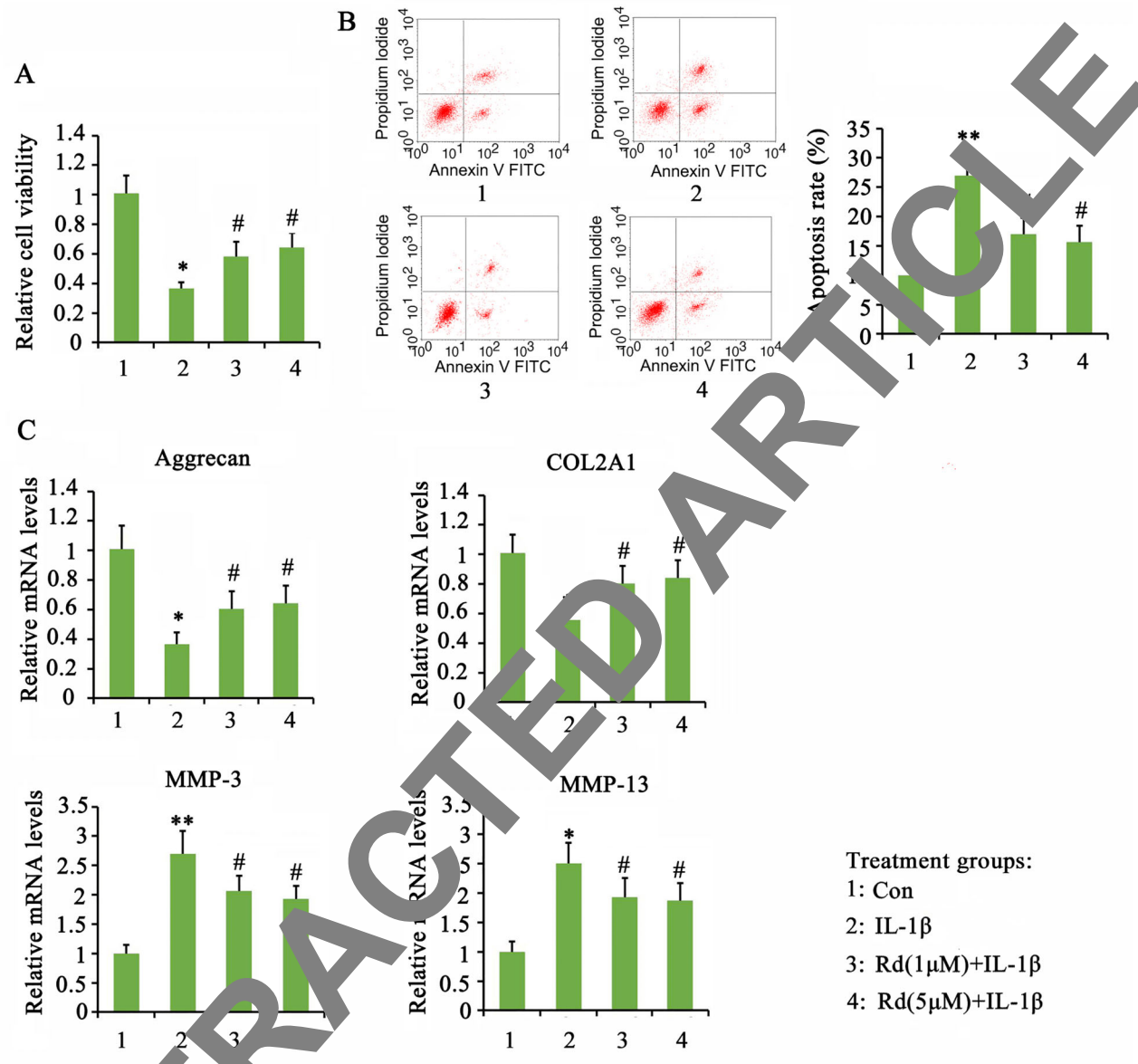

Figure 3. Ginsenoside Rd retarded interleukin (IL)-1β-induced degradation of nucleus pulposus (NP) chondrocytes. NP chondrocytes were treated with Rd prior to IL-1β. Cell viability and apoptosis rate were determined by CCK-8 (**A**) and flow cytometry analysis (**B**), respectively. The mRNA expression profiles in the cells were determined by PCR (**C**). Data are reported as means ± SD. *$P<0.05$ and **$P<0.01$ *vs* control; #$P<0.05$ *vs* IL-1β group (ANOVA).

($P<0.05$, Figure 3A). Pre-treatment with Rd (1 or 5 μM) conferred a protective effect against the reduction of cell viability ($P<0.05$ *vs* IL-1β group). The flow cytometry analysis showed that IL-1β-induced cell death ($P<0.01$) was attenuated with the treatment of Rd ($P<0.05$ *vs* IL-1β group, Figure 3B). After the treatment with IL-1β, the mRNA expressions of aggrecan and COL2A1 were reduced ($P<0.05$, Figure 3C), while MMP-3 ($P<0.05$) and MMP-13 ($P<0.01$) expressions increased in chondrocytes. However, pre-treatment with Rd reversed the changes in the expression of these genes ($P<0.05$ *vs* IL-1β group).

Rd inhibited IL-1β-induced activation of NF-κB mainly by increasing IL1RAP ubiquitination

The protein level of p65 in NP chondrocytes was not significantly changed by IL-1β alone or in combination with Rd pretreatment (Figure 4A). However, the phosphorylation level of p65 increased in NP chondrocytes with the IL-1β treatment ($P<0.01$, Figure 4A). Rd at the dosage of

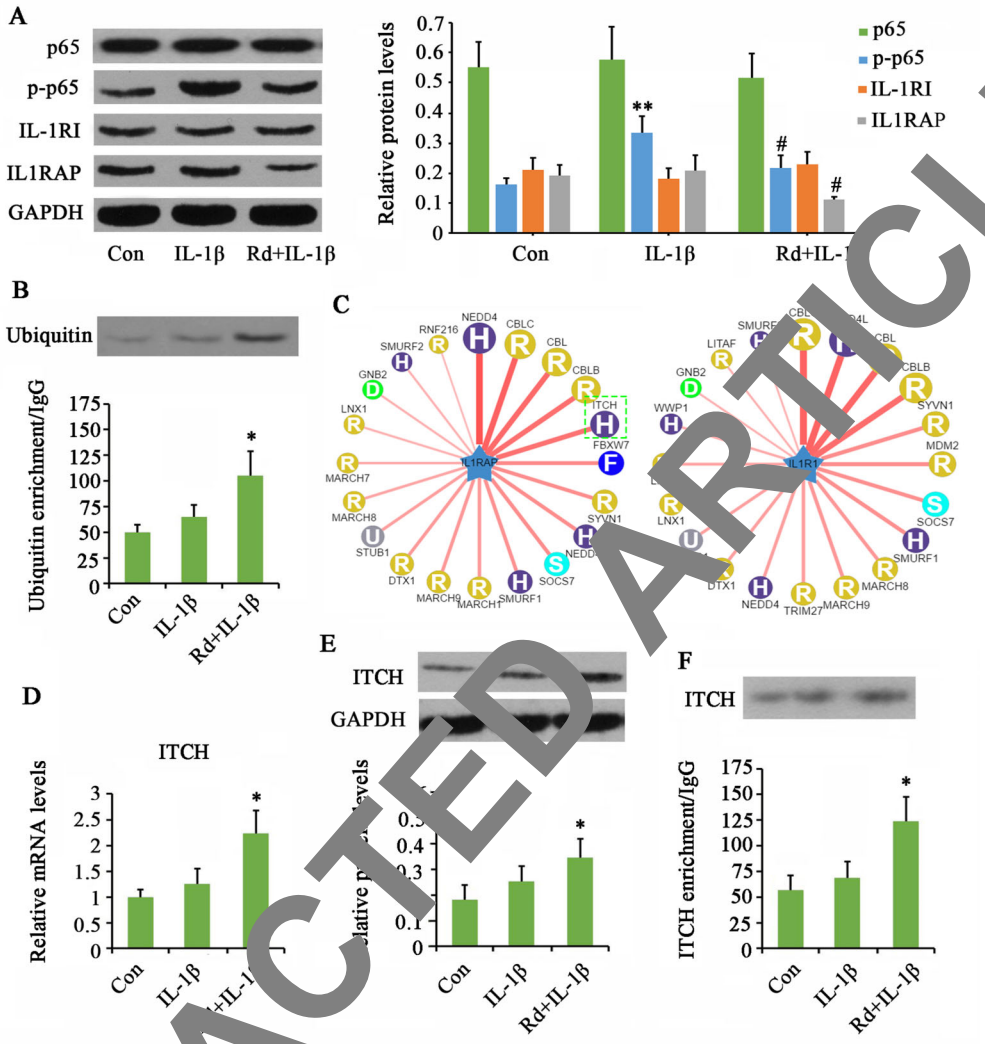

Figure 4. Ginsenoside Rd inhibited IL-1β-induced activation of NF-κB mainly by increasing IL1RAP ubiquitination. Nucleus pulposus chondrocytes were treated with Rd prior to IL-1β. **A**, Protein levels in the cells were assessed by western blot assay. **B**, Co-immunoprecipitation assay was performed to determine the change in the amount of ubiquitin that attached to IL1RAP. **C**, Bioinformatics analysis was performed to analyze the ubiquitinated ligases that target IL1RAP and IL-1RI (http://ubibrowser.ncpsb.org/ubibrowser/). The mRNA and protein levels of ITCH in the cells were determined by PCR (**D**) and western blot assays (**E**), respectively. **F**, The amount of ITCH attaching to IL1RAP was also determined by co-immunoprecipitation assay. Data are reported as means ± SD. **P < 0.01 and #P < 0.05 *vs* control; *P < 0.05 *vs* IL-1β group (ANOVA).

5 μM inhibited the increase in phosphorylation of p65 caused by IL-1β (P < 0.05 *vs* IL-1β group). IL-1-stimulated signal relies on two membrane-bound receptors: IL-1RI and IL1RAP. The present study measured the protein levels of IL-1RI and IL1RAP in NP cartilage cells after treatment with IL-1 alone or in combination with Rd. The protein levels of IL-1RI and IL1RAP were not significantly changed after treatment with IL-1β. However, treatment with Rd prior to IL-1β decreased the enrichment of IL1RAP in cartilage cells (P < 0.05 *vs* IL-1β group).

To determine the mechanism behind the down-regulation of IL1RAP, we initially performed RT-qPCR assay to assess the change in IL1RAP mRNA expression level. Results showed that IL1RAP mRNA expression did not significantly change by ginsenoside Rd (data not shown), suggesting that the reduction in IL1RAP by Rd was not associated with the pre-transcriptional regulation. We further performed co-immunoprecipitation assay to determine the change in the amount of ubiquitin that attaches to IL1RAP. IL1RAP was immunoprecipitated by the beads cross-linked with anti-IL1RAP antibody. The enrichment of ubiquitin in IL1RAP protein complex was evaluated by western blotting. Without IL-1β or ginsenoside Rd treatment, ubiquitin was almost undetectable in IL1RAP.

IL-1β moderately increased the enrichment of ubiquitin in IL1RAP protein complex. However, ginsenoside Rd treatment prior to IL-1β notably increased the amount of ubiquitin attached to IL1RAP ($P<0.05$, Figure 4B). Based on these data, we suggest that ginsenoside Rd caused a decrease in IL1RAP by increasing ubiquitination.

IL1RAP ubiquitination promoted by Rd was likely to occur through up-regulation of ITCH

Bioinformatics analysis was performed to analyze the ubiquitinated ligases that target IL1RAP and IL-1RI (http://ubibrowser.ncpsb.org/ubibrowser/). NEDD4 (neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase) and CBL (Cbl proto-oncogene) family proteins, including CBL, CBLB, and CBLC, as well as ITCH (itchy E3 ubiquitin protein ligase) were predicted to be most likely targeting IL1RAP (Figure 4C). CBLC, NEDD4L (neural precursor cell expressed, developmentally down-regulated 4-like, E3 ubiquitin protein ligase), CBL, and CBLB were predicted to be most likely targeting IL-1RI as well. As the protein level of IL-1RI was almost not changed by ginsenoside Rd, we hypothesized that IL1RAP ubiquitination promoted by Rd was through changing the expression and function of ITCH. Treatment with IL-1β only marginally increased the mRNA and protein levels of ITCH, but treatment with Rd prior to IL-1β increased the mRNA and protein levels of ITCH ($P<0.05$, Figure 4D and E). In addition, the amount of ITCH attaching to IL1RAP was increased with Rd treatment ($P<0.05$, Figure 4F).

Discussion

Various ginsenoside components show anti-inflammatory properties and have weak cytotoxicity. Kim et al. previously tested the effects of main ginsenosides (Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg3(S), Rh1(S), Rh2(S), and Rp1) and compound K on $CD4^+$ T cells (11). Results showed that treatment with 100 μM of ginsenosides promoted differentiation of T cells to regulatory T cells (Treg cells). Most ginsenosides did not affect cell viability, while Rg3(S), Rp1, and compound K showed cytotoxicity at 100 μM. Treg cells are a subpopulation of T cells that mainly function as immunosuppressive T cells by down-regulating the induction and proliferation of effector cells and modulate the immune system by maintaining self-tolerance and preventing autoimmune disease. While ginsenosides (e.g., Rd) inducing Treg differentiation represent an important anti-inflammatory mechanism, they have been found to directly inhibit the response of non-immune cells to inflammatory factors. Several studies demonstrated that ginsenosides attenuated the inflammatory response of articular chondrocytes upon inflammatory factors, therefore, they have been suggested as potential immunomodulating agents in the treatment of arthritis (7–10). To the best of our knowledge, for the first time, the present study showed that ginsenoside Rd inhibited the IL-1β-induced inflammatory action in intervertebral disc NP chondrocytes. Importantly, ginsenoside Rd at 100 μM had no toxic effect on NP chondrocytes.

The accumulation of inflammatory factors in the cell's surrounding microenvironment accelerates the degradation of chondrocytes and contributes to chondrocyte death, resulting in cartilage degenerative diseases. Although many plant extracts possess immunomodulatory effects, only a few have been studied in IVD chondrocytes. A recent study showed that morroniside, a phenolic component obtained from Mori Cortex, inhibited the increase in the levels of IL-1β, TNF-α and IL-6 in NP cells upon lipopolysaccharide and increased the autophagy-related proteins probably via PI3K/Akt/mTOR cascades (12). Besides, one study reported that *Chrysanthemum indicum* extracts conferred anti-inflammatory properties in ankylosing spondylitis mouse models by attenuating the activation of NF-κB (13). In our study, ginsenoside Rd not only inhibited the production of these inflammatory factors in NP chondrocytes in the presence of IL-1β but also hindered the increment in MMP-3 and MMP-13 expression with the increase of COL2A1 and aggrecan. Moreover, pre-treatment with Rd prevented NP chondrocytes from IL-1β-induced apoptosis. These data suggested that ginsenoside Rd had the capacity to attenuate the destructive effect of IL-1β on NP chondrocytes.

It has been established that over-activation of NF-κB signal mediated the detrimental effect of IL-1β (14). Blockage of the NF-κB signal using specific antagonists can attenuate the damage caused by IL-1β. However, NF-κB antagonists have seldom been used in clinical settings, considering their safety. NF-κB is a transcription factor that plays pivotal roles in many aspects of cellular processes such as inflammation, cell death, and cancer. The underlying mechanism of IL-1β activating NF-κB has been mostly elucidated (14). IL-1β-triggered pro-inflammatory signal that activates NF-κB is dependent on IL-1RI and IL1RAP (14). IL-1RI is the ligand-recognition receptor that binds IL-1β directly. Although IL1RAP does not bind IL-1β directly, its recruitment to IL-1RI following IL-1β stimulation is essential for the formation of an activated membrane receptor complex. The activated complex can recruit intracellular adaptor proteins and kinases, including MyD88, IRAK4, and IRAK1, and consequently activates downstream kinases IKK-α and IKK-β, which phosphorylate IκB proteins and lead to activation of NF-κB. Although various plant extracts have been reported to inhibit NF-κB activation by suppressing cell inflammatory responses (15), it remains unclear how they suppress NF-κB activation. The present study showed that ginsenoside Rd decreased IL1RAP protein level but not the mRNA level in NP chondrocytes. Thus, ginsenoside Rd likely promoted IL1RAP ubiquitination, resulting in IL1RAP down-regulation. In fact, the amount of ubiquitin attaching to

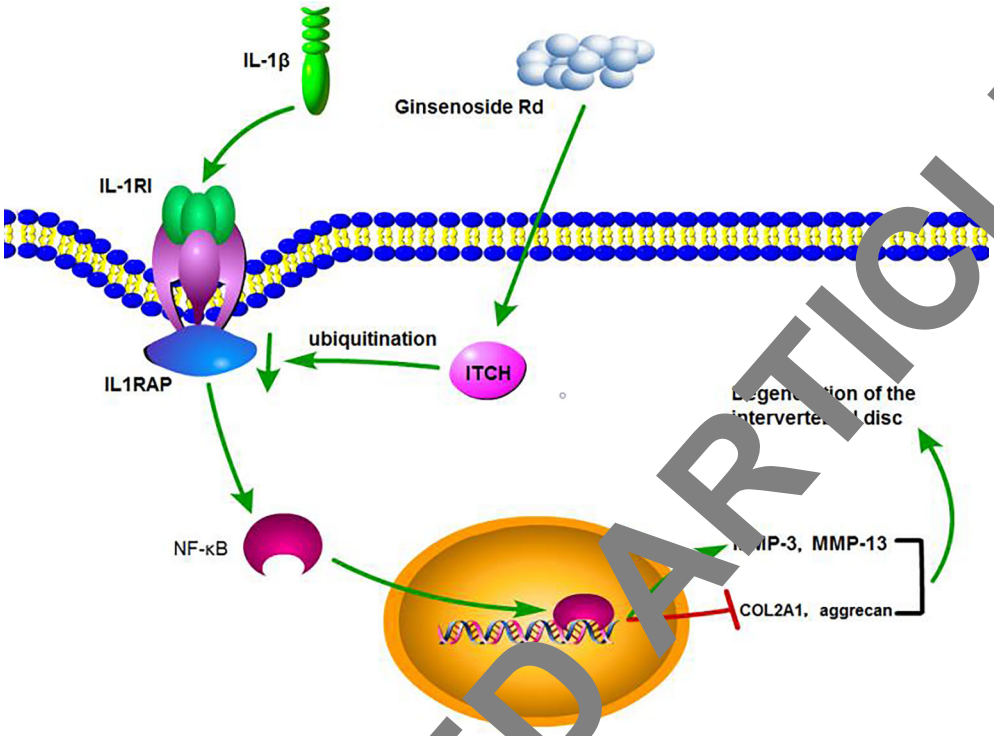

Figure 5. The protective mechanism underlying ginsenoside Rd against interleukin (IL)-1β-induced degradation and death of chondrocytes. The protective effect is associated with Rd driving IL1RAP ubiquitination by upregulating ITCH. IL1RAP ubiquitination further blocks the stimulation of NF-κB by IL-1β. The inhibition of NF-κB is responsible for the suppressed inflammatory response of chondrocytes to IL-1β.

IL1RAP protein was notably increased with ginsenoside Rd treatment.

To further understand the mechanism underlying Rd-induced IL1RAP ubiquitination, we initially performed bioinformatics analysis to identify the ubiquitinated ligases that targeted IL1RAP. Among these, most likely targeting IL1RAP, ITCH was upregulated by ginsenoside Rd. In addition, ginsenoside Rd increased the amount of ITCH attaching to IL1RAP. These results suggested that IL1RAP ubiquitination promoted by Rd is likely to occur through up-regulation of ITCH. ITCH activity has been reported to be regulated by the JNK pathway (16,17), and this signaling is further under the modulation of Rd and many other components of ginsenoside (18–21). However, the regulatory effects of ginsenoside components on the JNK pathway varied in different circumstances. Further study is needed to elucidate the mechanism underlying the regulatory effect of Rd on ITCH expression and activity.

The present study identified the protective effect of Rd against IL-1β-induced degradation and death of chondrocytes. The protective effect was associated with Rd driving IL1RAP ubiquitination by upregulating ITCH. IL1RAP ubiquitination further blocked the stimulation of NF-κB by IL-1β. The inhibition of NF-κB was responsible for the suppressed inflammatory response of chondrocytes to IL-1β. This novel mechanism is displayed in Figure 5.

Acknowledgments

This study was funded by Project of Teaching Quality and Teaching Reform of Xinhua College of Sun Yat-sen University in 2018: Biochemistry, a Fine Resource Sharing Course, 2018JP002.

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