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Metformin protects chondrocytes against IL-1β induced injury by regulation of the AMPK/NF-κB signaling pathway

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Received September 3, 2020, accepted October 3, 2020

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Pharmazie 75: 632-636 (2020) *doi: 10.1691/ph.2020.0762*

Osteoarthritis (OA) is a common joint disorder characterized by degeneration and inflammation of the articular cartilage. The etiology of OA is complex, and there is no effective drug for the treatment currently. Metformin, the first-line drug for type 2 diabetes mellitus, has been reported to play an essential role in a variety of diseases; however, whether it could be used in OA therapy remains unclear. In this study, we used interleukin-1β (IL-1β) to mimic the pathophysiology of OA to explore the function and the underlying mechanism of metformin on OA. In our study, cell viability was measured using cell counting kit-8 assay, expressions of crucial factors involved in the extracellular matrix (ECM) metabolic, proinflammatory response, cell apoptosis, and nuclear factor κB(NF-κB) pathway were analyzed using western blot analysis and immunofluorescence staining. We found that metformin increased the proliferation of the cells, alleviated IL-1β-induced ECM metabolic imbalance and proinflammatory cytokine production, and exerted anti-apoptosis activity in ATDC5 cells. Furthermore, the results showed that metformin blocked the NF-κB pathway in IL-1β-induced ATDC5 cells *via* activation of AMP-activated protein kinase (AMPK). These results indicated that metformin protected chondrocytes against IL-1β-induced injury, possibly by regulation of the AMPK/NF-κB signaling pathway. It may have the potential as a novel drug for OA treatment.

1. Introduction

Osteoarthritis (OA) of the knee is an age-related, chronic disease characterized by cartilage abrasion and degradation, osteophyte formation, subchondral bone remodeling, and low-grade inflammation (Glyn-Jones et al. 2015; Taruc-Uy and Lynch 2013). As a common disease that features joint pains, limited movement, and joint deformity, OA seriously affects the quality of life and causes great social pressure. Various factors participate in the pathogenesis of OA, including aging, sex, obesity, muscle weakness, trauma history, and genetic predisposition (Silverwood et al. 2015). However, the overall cause of OA is still poorly understood. Currently, there are no especially effective drug therapies for this kind of disease; existing drugs such as NSAIDs do not effectively ameliorate symptoms while displaying severe adverse effects (Bijlsma et al. 2011). Hence joint replacement becomes the only choice eventually (Bijlsma et al. 2011; da Costa et al. 2017). Therefore, the development of effective and safe measures to aid in OA management is urgent.

Chondrocytes are the only resident cell type present in articular cartilage, which constitutes only about 5% of the total cartilage volume, and the remainder is occupied by an extensive extracellular matrix (ECM) (Wang et al. 2011). Several biomechanical or biochemical events may damage chondrocytes, thereby inducing their apoptosis and disrupting the balance between the anabolism and catabolism of the ECM (Wang et al. 2011). It is generally believed that inflammation is a vital driver in the metabolic disorders and enhanced catabolism of tissue in the OA joint (Goldring and Goldring 2004). And inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) play vital roles in the progression of OA (Loeser 2006).

Metformin, a common oral hypoglycemic drugs used to treat type 2 diabetes, has additionally been identified to play a crucial role in regulating fat (Yerevanian and Soukas 2019), protecting from cardiovascular disease (Ratner et al. 2005), tumors (Pernicova and Korbonits, 2014) and other metabolic disorders. But its effect on bone metabolism is still controversial. Many clinical data

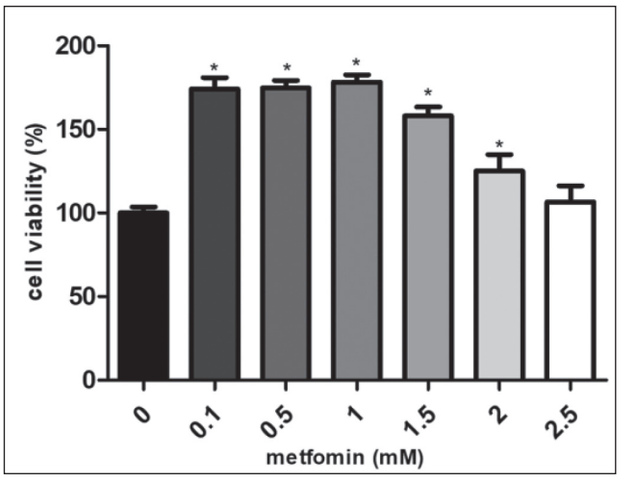

Fig. 1: Effects of metformin on ATDC5 cell viability. ATDC5 cells were treated with metformin at different concentrations (0.1, 0.5, 1, 1.5, 2, 2.5 mM) for 24 h. Cell viability detected using Counting Kit-8 assays (CCK8). CCK-8: Cell Counting Kit-8. Data are presented as mean±standard deviation of the mean (SD). * $P<0.05$ *vs*. 0 metformin.

confirmed that metformin reduces the risk of fractures in diabetics; however, a few studies reported that metformin increases bone fraction (Meier et al. 2016; Vestergaard et al. 2005). And there is limited research about the effects of metformin on the risk of OA. Recently, metformin was found to possess an anti-inflammatory effect (Han et al. 2019; Horiuchi et al. 2017). However, the mechanism of its anti-inflammatory activities has not been fully elucidated (Han et al. 2019). The present study aimed to investigate whether metformin could protect chondrocytes from inflammatory injury induced by IL-1β.

2. Investigations and results

2.1. Effect of metformin on cell viability

Cell viability of ATDC5 cells treated with metformin was analyzed using the CCK-8 assay. The results indicated that metformin could increase the proliferation of the cells in the concentration range of 0.1–2.5 mM. Moreover, metformin at the concentration of 1 mM most significantly promoted cell proliferation in ATDC5 cells (Fig. 1). Hence, this concentration (1 mM) was selected in subsequent experiments.

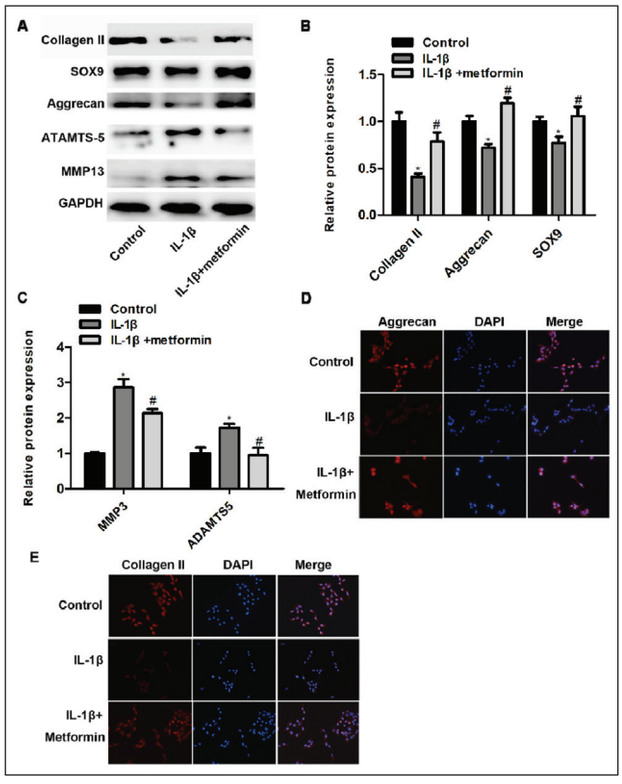

Fig. 2: Metformin alleviated IL-1β induced extracellular matrix degradation. (A) Western blot assay was performed to detect the protein expression level of Aggrecan, Collagen II, SOX9, MMP13, ADAMTS5. (B) (C)The Image-J software was used to quantified relative protein expression, and results are the representative of three independent experiments. (D)(E) immunofluorescence staining of aggrecan and collagen II, magnification×200. IL-1β: interleukin-1β, MMP13: matrix metallopeptidase 13, SOX9: SRY-Box Transcription factor 9, ADAMTS5: a disintegrin and metalloproteinase with thrombospondin motifs 5. Data are presented as mean±standard deviation of the mean (SD). *P< 0.05 *vs.* control group. # P<0.05 *vs.* IL-1β group.

2.2. Metformin attenuates the IL-1β-induced chondrocyte catabolism and inflammatory response in ATDC5 cells

In our study, IL-1β was applied to chondrocytes as the *in vitro* osteoarthritis model. Western blot analysis indicated that the protein expression of Collagen II, Aggrecan, and SOX9 following treatment with IL-1β was significantly suppressed, while MMP3 and ADAMTS5 were upregulated (Fig. 2A-C). In addition to suppresses the synthesis of major ECM components, previous studies have demonstrated that IL-1β can stimulate inflammatory cytokine production in OA pathology. In our study, the protein levels of IL-6 and TNF-a, two major inflammatory cytokines in OA, were also obviously increased with the stimulation of IL-1β, while co-stimulation with metformin could partly reverse these IL-1β effects (Fig. 3A and 3B). Furthermore, we measured the cartilage-specific gene by immunofluorescence staining, consistently, the protein levers of Collagen II, Aggrecan were dropped (Fig. 2D and E). The above data revealed that metformin exerts anti-catabolic and anti-inflammatory effects on IL-1β-induced chondrocytes.

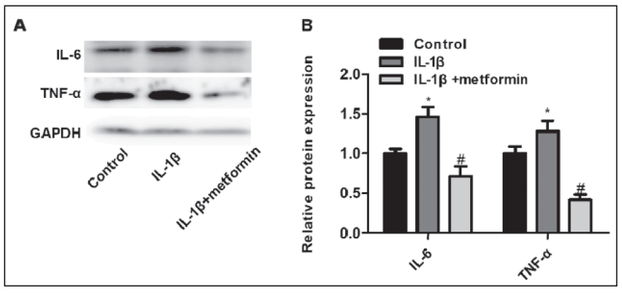

Fig. 3: Effects of metformin on the expression of inflammatory factors in IL-1β-treated ATDC5 cells. (A) Western blot assay was performed to detect the protein expression level of IL-6 and TNF-α. (B) The Image-J software was used to quantified relative protein expression, and results are the representative of three independent experiments. IL-6: interleukin-6, TNF-α: tumor necrosis factor-α. Data are presented as mean±standard deviation of themean (SD). *P< 0.05 *vs.* control group. # P<0.05 *vs.* IL-1β group.

2.3. Metformin attenuates IL-1β-induced chondrocyte apoptosis

Next, the impact of metformin on IL-1β triggered cell apoptosis was explored. The CCK-8 analysis suggested that cell viability prominently reduced when treated with IL-1β, and this effect was partly reversed by co-treatment with 1 mM metformin (Fig. 4A). Chondrocyte numbers were also significantly reduced following IL-1β treatment, and metformin substantially prevented chondrocytes from undergoing IL-1β-induced morphology change and death (Fig. 4D). We further detected the protein expression of apoptosis regulatory markers. Consistent with prior studies, Western blot analysis showed that IL-1β treatment significantly increased Bax/Bcl-2 ratio. However, metformin remarkably inhibited IL-1β-induced Bax overexpression but enhanced Bcl-2 expression (Fig. 4B and 4C). These results suggested that metformin exerts anti-apoptosis effects on IL-1β-treated chondrocytes.

2.4. Metformin exerts protective effects on IL-1β-induced chondrocytes via regulation of the AMPK/NF-kB signaling pathway

Previous data have shown that NF-κB pathways play a crucial role in the regulation of inflammatory mediators associated with OA. It is well known that metformin is an AMPK activator, and NF-κB is downstream signaling of AMPK. Hence we hypothesized that the anti-inflammatory effect of metformin in chondrocytes might be due to the inhibition of the NF-κB signaling cascades by the activation of AMPK. Through western blot, we found that in addition to activating AMPK, metformin attenuated phosphorylation of the NF-κB subunit in response to IL-1β (Fig. 5A-D). Immunofluorescence analysis further indicated that the nuclear translocation of NF-κB caused by IL-1β was inhibited by metformin (Fig. 5E). These data indicated that the inhibitory effects of metformin on the inflammation and catabolism of the chondrocytes might be mediated by the AMPK/NF-kB pathway.

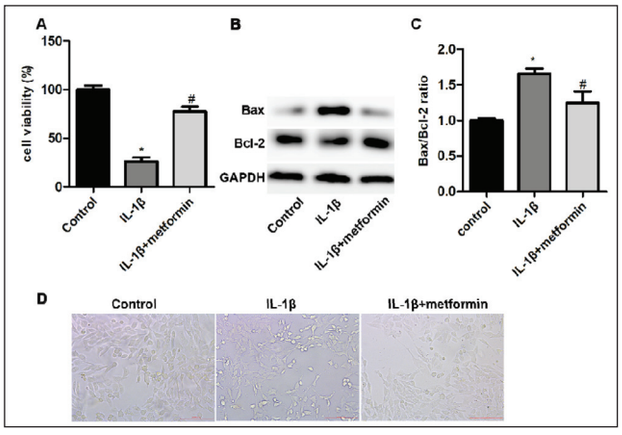

Fig. 4: Metformin alleviates IL-1β-induced cell apoptosis in ATDC5 cells. (A) After stimulation with IL-1β (10 ng/ml) for 24h in the presence or absence of metformin (1 mM), cell viability was detected by the CCK-8 assay. (B) Western blot assay was performed to detect the protein expression level of Bax and Bal-2. (C) The Image-J software was used to quantified relative protein expression, and results are the representative of three independent experiments. (D) Morphology of ATDC5 cells in monolayer culture, magnification ×200. Bal-2: B-cell lymphoma 2, Bax: BCL2-associated X protein. Data are presented as mean±standard deviation of the mean (SD). *P< 0.05 *vs.* control group. # P<0.05 *vs*. IL-1β group.

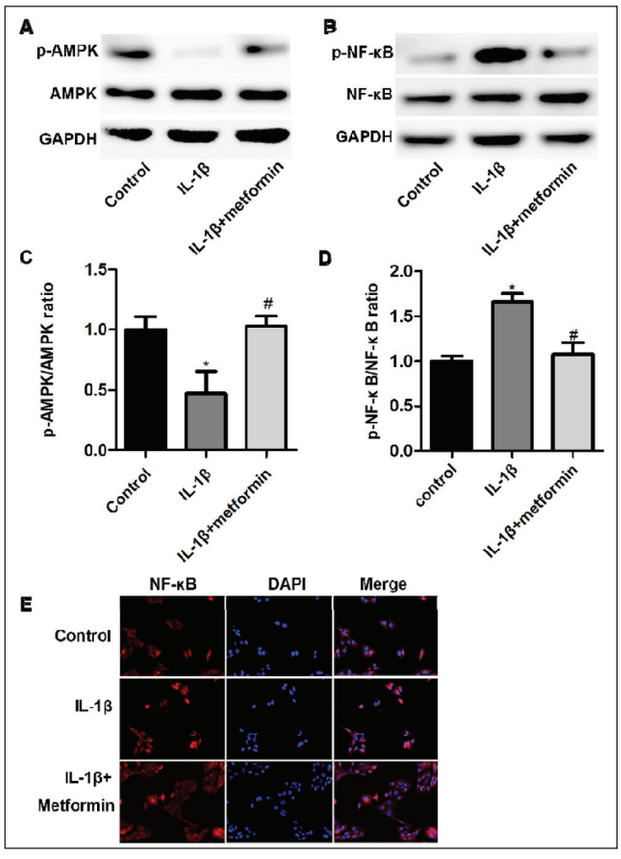

Fig. 5: Metformin inhibits IL-1β -induced NF-κB pathway activation via activation of AMPK in ATDC5 cells. (A)(B)Western blot assay was performed to detect the protein expression level of NF-κB, p- NF-κB, AMPK, and p-AMPK, (C) (D)The Image-J software was used to quantified relative protein expression, and results are the representative of three independent experiments. (E) NF-κB nuclear translocation was assessed by immunofluorescence staining for NF-κB (red), Cell nuclei were detected by DAPI (blue), magnification×200. NF-κB: nuclear factor kappa-B, AMPK: Adenosine 5-monophosphate (AMP)-activated protein kinase, p-: phosphorylated. Data are presented as mean±standard deviation of the mean (SD). *P< 0.05 *vs.* control group. # P<0.05 *vs*. IL-1β group.

3. Discussion

OA is a common form of arthritis, characterized by articular cartilage breakdown. Cartilage is composed of chondrocytes embedded in cartilage ECM, and ECM is produced by chondrocytes (Hashimoto et al. 2008). During the progression of OA, chondrocytes experience increased apoptosis, production of pro-inflammatory factors, and degradation of the ECM. Therefore, targeting of chondrocyte injury is thought to be a valuable strategy for developing effective therapies for OA.

IL-1β, a representative pro-inflammatory cytokine, has been reported to exert a potent effect on the articular cartilage destruction (Attur et al. 1998). Elevated IL-1β levels have been found in the synovial fluid of OA patients, and IL-1β have been shown to inhibit the synthesis of major extracellular matrix (ECM) components Collagen II and proteoglycans (Sharif et al. 2004). On the other hand, IL-1β increases the production of catabolic enzymes, such as matrix metalloproteinases (MMPs), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Bondeson et al. 2008; Feng et al. 2017). It has been demonstrated that MMP-3 is mainly responsible for the degradation of ECM as it is efficiently and irreversibly cleaved Collagen II, Aggrecan, and other extracellular matrix macromolecules in the pathogenesis of the OA. Besides, ADAMTS5 as the key aggrecanase plays a major role in the degradation of Aggrecan (Bondeson et al. 2008; Majumdar et al. 2007). In the present study, we used IL-1β to establish a cellular model of OA. As expected, the expression of Collagen II, Aggrecan, and SOX9, decreased in response to IL-1β, while MMP3, ADAMTS5 was upregulated and metformin reversed this effect. Besides, IL-6 and TNF-α have been suggested to play pivotal roles in many inflammatory diseases, including OA (Zhang et al. 2013; Zhou et al. 2018). They could activate macrophages, which subsequently synthesize a wide array of proinflammatory chemokines to maintain inflammation in OA development and progression (Feldmann et al. 1996). In our study, we found that the induction of TNF-α and IL-6 production by IL-1β was abolished by metformin. These results suggested that metformin may play a protective role in OA disease, including anti-catabolic and anti-inflammatory effects.

Chondrocyte apoptosis is one of the main features of cartilage degeneration (Sharif et al. 2004). Several studies have reported that chondrocyte apoptosis is associated with the cellular changes in cartilage and loss of articular cartilage (Heraud et al. 2000). It has been reported that metformin exerts anti-cancer effects *via* inducing apoptosis in tumor cells; on the other hand, metformin also shows beneficial effects against apoptosis in many cells, including hepatic cell (Geng et al. 2020), testicular cells (Liu et al. 2019) and cardiac cells (Wang et al. 2019). However, less is known about whether and how metformin participates in IL-1β-induced chondrocyte apoptosis. Our results showed that IL-1β significantly increased Bax/Bcl-2 ratio in ATDC5 cells, which was consistent with previous studies (Wang et al. 2015). It is well known that Bax and Bcl-2 are regulatory markers of apoptosis. The upregulated level of Bax induces apoptosis, whereas Bcl-2 exhibits anti-apoptotic actions (van Delft and Huang 2006). Our present results indicated that metformin alleviates the anti-apoptotic effect of IL-1β by decreasing the ratio of Bax to Bcl-2.

We further investigated the molecular mechanisms by which metformin suppresses IL-1β-induced chondrocyte injury. As we all know, metformin is an AMPK activator. The AMPK protein exists as heterotrimeric complexes comprising an α-catalytic subunit with two regulatory β and γ subunits. And activation of AMPK requires phosphorylation at Thr-172 within the catalytic α subunit (Grahame Hardie 2014). AMPK is a master regulator of energy homeostasis, allowing the inhibition of energy (e.g., ATP) consuming cellular processes, and the activation of energy-producing processes and dysregulation of AMPK has been implicated in a variety of human diseases and aging. Furthermore, activation of AMPK has been shown to have a strong anti-inflammatory effect in several animal models, such as diabetic neuropathy, the severity of acute lung injury, and arthritis (Xiang et al. 2019). Its role in osteoarthritis remains controversial. Reduced phosphorylated AMPK was observed in mouse surgical instability-induced and human OA knee cartilage.

And in aged mouse knee cartilage, AMPK phosphorylation was also reduced (Zhou et al. 2017). However, certain reports indicated that the chondrocyte-specific ablation of AMPK has no effect on OA development (Yang et al. 2016). In our study, the protein expression of p-AMPK decreased with the stimulation of IL-1β; this effect was significantly reversed by co-treatment with 1 mM metformin.

Nuclear factor-κB (NF-kB) is a critical transcription factor involved in varied biological processes, including inflammation and apoptosis (Oeckinghaus and Ghosh 2009). Previous studies have suggested that NF-κB plays an important role in the production of several inflammatory mediators in chondrocytes induced by IL-1β (Cheleschi et al. 2018). NF-kB is downstream signaling of AMPK (Okayasu et al. 2008), and there are emerging results suggested that AMPK signaling can inhibit the NF-κB-induced inflammation inflammatory responses (Xiang et al. 2019). In an inactive state, NF-κB combines with inhibiting NF-κB proteins (IκBs) and localized to the cytoplasm (Oeckinghaus and Ghosh 2009). In the presence of IL-1β, the phosphorylation of NF-κB is induced by IL-1β, activated NF-κB is separated from IκB and translocated to the nucleus, which in turn induces the expression of inflammatory mediators and matrix-degrading enzymes (Okayasu et al. 2008; Rigoglou and Papavassiliou 2013). In our study, we found that metformin blocked the phosphorylation of NF-κB and the translocation from the cytosol to the nucleus induced by IL-1β *via* activation of AMPK. Hence we suspected that the beneficial effect of metformin on OA chondrocytes metabolism, probably due to the modulation of the AMPK-NF-κB pathway.

Collectively, the present results suggested that metformin exerted anti-catabolic, anti-inflammatory, and anti-apoptosis effects in the IL-1β-induced ATDC5 chondrocytes through AMPK/NF-κB signaling pathway. These findings demonstrate that metformin has potential as a novel drug for the treatment of OA. However, the present study is a preliminary study, and further studies are needed to validate the role of metformin in OA.

4. Experimental

4.1. Reagents and antibodies

Metformin was purchased from Beyotime Institute of Biotechnology (Shanghai, China). IL-1β was obtained from MedchemExpress (Monmouth Junction, NJ, USA). Antibodies against Collagen II, Aggrecan, SOX9, MMP3, ADAMTS5, NF-κB, p-NF-κB, Bax, and Bcl-2 were obtained from Abcam (Cambridge, UK). p-AMPK, AMPK, IL-6, TNF-a antibodies were from Cell Signaling Technology (Beverly, MA, USA), and the GAPDH antibody was from ProteinTech Group (Shanghai, China).

4.2. Cell culture and differentiation

The murine ATDC5 chondrocyte cell line was purchased from the Chinese Academy of Sciences, the Science Cell Bank of the Type Culture Collection (Shanghai, China). And maintained in the complete medium of DMEM/F12 (HyClone) supplemented with 5% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), in an incubator at 37 °C with 5% CO_2. After cell attachment, insulin, transferrin, selenite (ITS, Sigma), and ascorbate (sigma) was added to the medium for 14 days to form chondrogenic ATDC5 cells. The culture medium was changed every two days, and cells were passaged until confluence was achieved 80–90%.

4.3. Cell viability assay

Cell viability was assessed by cell counting kit-8 (CCK-8; Medchem Express) assay. ATDC5 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. After cell attachment, cells were treated with IL-1β or metformin for 24 h. Then 10 μl CCK-8 solution was added; after 1 h, optical density was measured using a microplate reader (Thermo Fisher Scientific, Inc.) at 450 nm.

4.4. Western blotting

After stimulation with metformin (1 mM) for 24 h in the presence or absence of IL-1β (10 ng/mL), Total proteins from the chondrocytes were extracted from chondrocytes using ice-cold RIPA lysis buffer, and the concentration of proteins was quantified using the BCA protein assay kit (Beyotime). After that, equal amounts of total proteins were subjected to 10% SDS-PAGE or 12% SDS-PAGE and electroblotted onto a PVDF membrane. Membranes were blocked with 5% powdered non-fat dried milk at room temperature for 1 h and incubated with appropriate primary antibodies at 4 °C overnight. After washing three times in TBST (each for 10 min), the membranes were incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were visualized with enhanced chemiluminescence reagents (Millipore). All tests were repeated three times. GAPDH was used as an internal control.

4.5. Immunofluorescence staining

ATDC5 cells were plated on glass coverslips and grown for 24 h. After cell attachment, cells were stimulated with metformin (1 mM) for 24 h in the presence or absence of IL-1β (10 ng/mL), the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized by treatment with 0.5%Triton X-100 for 20 min at room temperature. Following blocking with BSA goat serum for 30 min, cells were incubated with primary antibodies against Aggrecan (1:50), Collagen II (1:200), and NF-κB (1:500) overnight 4 °C. The second day, samples were incubated with corresponding secondary antibodies for 1 h, and nuclei were stained with DAPI for 5 min. The images were observed by a fluorescence microscope (Axiovert 100M Zeiss, Germany).

4.6. Statistical analysis

All the experiments were repeated at least three times, and the data are presented as the mean±standard deviation. Prism v.5 (GraphPad Software) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to analyze the data. $P<0.05$ denotes a statistically significant difference.

Acknowledgments: This research was supported by Key Research and Development Plan Project of Shandong Province (grants no. 2016 GSF201025, 2016GGH3118), the National Natural Science Foundation of China (grant no. 81370892) and the Shandong Taishan Scholars Specially-invited Expert Plan.

Conflicts of interest: None declared.

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