Metformin Combined with Alendronate Ameliorates Osteoarthritis by Attenuating RANKL-induced Bone Resorption and Protecting Cartilage Against Degradation

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Abstract

The purpose of the present study was to investigate the effect of metformin combined with alendronate on subchondral bone and cartilage degeneration in a destabilization of the medial meniscus (DMM)-induced osteoarthritis (OA) mouse model. There were lower OARSI scores and fewer TRAP-positive cells in DMM mice after treatment with metformin combined with alendronate. In cartilage, metformin inhibited the expression of MMP3 and MMP13 in IL-1 β -stimulated OA chondrocytes and increased the expression of Sox9 and aggrecan. In parallel, metformin also inhibited cartilage matrix degradation, as shown by Alcian blue staining and SO/FG staining in ATDC5 cells and cartilage tissues. Moreover, metformin and alendronate downregulated the levels of specific genes and proteins (NFATc1, c-Fos, DC-STAMP, TNFRSF11, CTSK), thereby inhibiting osteoclast differentiation in vitro. Mechanistically, metformin increased the expression of phospho-AMPK α 1, attenuated the phosphorylation of NF- κ B, and reduced reactive oxygen species (ROS) levels. Metformin combined with alendronate may be a useful candidate for attenuating early-stage OA. This would be an ideal drug/drug combination for treating osteoarthritis in the presence of osteoporosis combined with T2D.

Introduction

Osteoarthritis (OA) is a chronic joint disease characterized by cartilage degeneration and subchondral bone remodeling [1]. The development of osteoarthritis is associated with several factors, including aging, joint inflammation, and joint instability, and is driven by biological pathways within the joint and biomechanical factors[2]. One recent meta-analysis suggested that patients with type 2 diabetes (T2D) were more likely to develop knee OA than non-diabetic patients[3]. Moreover, the biomechanical quality of bone is compromised in diabetic patients, so T2D often cooccurs with osteoporosis[4]. Alendronate, which inhibits bone resorption, is therefore the main choice for treating osteoporosis. In a large retrospective case-control study, the incidence of T2D was reduced in patients with osteoporosis who were treated with alendronate[5]. Metformin is the first-line therapy for diabetes mellitus type 2 when obesity is present. Due to its protective effect on the skeletal system, metformin is used preferentially for treating T2D in patients with osteoporosis.

In a cohort study, patients with OA and T2DM were treated with a combination of COX-2 inhibitors and metformin, and these patients had a lower rate of joint replacement surgery than those who were not treated with the combination[6]. This outcome may be because the combination treatment reduced the associated proinflammatory factors more than in patients who were not treated with metformin. Therefore, metformin may be a potential candidate for the development of effective treatments for OA. The antidiabetic effect of metformin is mainly achieved by activating AMP-activated protein kinase (AMPK) through the stimulation of Thr-172 phosphorylation on AMP-activated protein kinase α 1 (AMPK α 1)[7]. AMPK is a chondrocyte bioenergy sensor that has been suggested to be involved in OA development[8], and its activity is decreased in human knee OA chondrocytes and cartilage[9]. Knockout of AMPK α 1, the catalytic α subunit of AMPK, in mice accelerates the progression of osteoarthritis induced by destabilization of the medial meniscus surgery[10]. These findings indicated that maintaining

AMPK activity in chondrocytes may be important in delaying cartilage damage. Recently, metformin has been proven to have anti-inflammatory effects in treating OA. Some studies have shown that metformin enhances AMPK phosphorylation in chondrocytes and subsequently delays OA development in a mouse destabilization of the medial meniscus (DMM) model[11]. When AMPKa1 was knocked out, the chondroprotective effect of metformin was lost[11]. This finding means that a chondrocyte-targeted version of metformin can be used to treat OA. Furthermore, it has been shown that AMPK negatively regulates the differentiation and function of osteoclasts[12].

The subchondral bone plays a significant role in joint homeostasis and OA development[13]. The structural alterations in subchondral bone in OA are thought to enhance crosstalk with articular cartilage and therefore accelerate the progression of OA[14]. Animal studies have shown that changes in subchondral bone, such as increased osteoclast activity and bone remodeling, might occur at the same time as changes in cartilage[15]. In a study in which meniscectomized mice were treated with OPG, an inhibitor of osteoclastic bone resorption activity, cartilage destruction in the mice was abrogated[16]. Alendronate, a bisphosphonate, also limits the progression of OA by enhancing subchondral bone mass by inhibiting osteoclasts and bone resorption[17]. These studies suggest that restoring abnormal subchondral bone reconstruction by inhibiting osteoclastic bone resorption in the early stages of OA could be a therapeutic strategy for OA. However, there is a risk that long-term use of alendronate may alter bone strength due to excessive inhibition of bone transformation[18].

In the present study, we investigated the therapeutic effect of low concentrations of alendronate combined with metformin and administered at low frequency in a murine model of OA induced by surgical destabilization of the medial meniscus. We found that low concentrations of alendronate counteracted DMM-induced subchondral bone loss and cartilage degeneration and that these effects were enhanced when alendronate was combined with metformin. Furthermore, the mechanism by which metformin affected the AMPK signaling pathway was analyzed. In addition, metformin combined with alendronate may delay the progression of osteoarthritis in patients with T2D combined with osteoporosis.

Method

Mice

OA was induced in 8-week-old male C57BL/6 mice by destabilization of the medial meniscus (DMM) in the right knee, followed by random allocation into a control group and two treatment groups: treatment with alendronate alone or alendronate plus metformin (n=5 per group). The day after surgery, Group A received 2 intraperitoneal injections of alendronate (APExBIO, CAS No. 121268-17-5) weekly for 8 weeks at a dose of 0.1 mg/kg. Group B received alendronate plus 205 mg/kg metformin (CSNpharm, CAS No. 627-24-9) in the same manner.

Cell culture

We purchased the murine ATDC5 cell line from Shanghai Jining Industrial Co. The cells were cultured in a complete medium consisting of DMEM/F12 (HyClone), 1% penicillin/streptomycin (Sigma), and 10% fetal bovine serum (CellMax). We changed the medium every 2 days. The cells were passaged when the cells were 80%-90% confluent.

Bone marrow monocytes (BMMs) were isolated from the tibia and femur marrow of C57BL/6 mice. BMMs were maintained in complete medium consisting of alpha-modified minimal essential medium (α -MEM) (HyClone) containing 10% fetal bovine serum (vwr), 1% penicillin/streptomycin (Sigma) and 25 ng/mL macrophage-colony-stimulating factor (M-CSF) (LifeTein, New Jersey, USA). Afterward, the BMMs were stimulated with 50 ng/mL receptor activator of nuclear factor- κ B ligand (RANKL) (LifeTein, New Jersey, USA) and 25 ng/mL M-CSF.

Cell treatment and CCK-8 assay

We used different concentrations of metformin (0, 2, 4, and 5 mmol/L) to treat the murine ATDC5 cell line with or without 10 ng/mL recombinant murine interleukin (IL)-1 β (PeproTech, Rocky Hill, NJ, USA).

BMMs were cultured with varying concentrations of alendronate (0, 62.5, 125 nmol/L) or metformin (0, 62.5, 125 μ mol/L) in the presence of 100 ng/mL RANKL.

For CCK-8 assays, the cells were seeded into 96-well plates. After adherence, the cells were treated with different concentrations of metformin or alendronate. Two days later, 10 μ L of CCK-8 solution (JoyTech Bio, Zhejiang, China) was added to the wells and incubated for 2 h. The optical density at 450 nm was measured using a microplate reader.

High-density culture of the murine ATDC5 cell line

EDTA-trypsin (Gibco) was used to digest the cells, and the cells were resuspended at a concentration of 2×10^6 /mL. Then, 10 μ L of the cell suspension was added dropwise at hourly intervals to a 24-well plate, 4 times in total. Then, 500 μ L of complete medium was added to each well with or without 10 ng/mL IL-1 β and incubated for 6 days. Moreover, different concentrations of metformin (0, 2, 4, and 5 mmol/L) were added to the cultured cells. The medium was changed every other day. The chondrogenic matrix was examined by Alcian blue staining. The stained area was scanned by the scanner and measured with ImageJ.

Organoid culture

The “relatively normal” cartilage tissue from the tibial plateau of a total knee replacement patient was cut into small pieces and cultured in DMEM/F12 supplemented with 10% FBS with various concentrations of metformin (0, 2, 4, and 5 mmol/L with or without IL-1 β (10 ng/mL)). The cartilage pieces were collected at 24, 48, and 72 h. After 16 h of fixation with 4% paraformaldehyde, the sample was dehydrated through a graded series of ethanol, cleared, and embedded in paraffin. Then, 5- μ m-thick sections were cut and collected on glass slides. Integrated optical density

values of proteoglycan content were determined by Safranin O-fast green and immunohistochemical staining.

The efficacy of metformin and alendronate on osteoclasts

BMMs were seeded at a density of 8×10^3 cells/well in a 96-well plate with complete alpha-MEM containing RANKL (50 ng/mL) in the presence of metformin (0, 62.5, and 125 μ mol/L) or alendronate (0, 62.5 and 125 nmol/L) for 7 days. The medium was changed every 2 days. After osteoclasts formed, the differentiated osteoclasts were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) (JoyTech Bio, Zhejiang, China). We evaluated the differentiation of osteoclasts by counting TRAP-positive multinucleated cells (MNCs) that contained 3 or more nuclei.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After 7 days of stimulation with metformin (0, 2, 4, 5 mmol/L) in the presence or absence of IL-1 β , we extracted total RNA from chondrocytes with TRIzol reagent (Takara, Japan). The RNA concentration was determined using NanoDrop one at 260 nm. Then 1 μ g of total RNA was reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (Takara, Japan). The expression of target genes was examined using a QuantiTect SYBR Green PCR kit (Takara, Japan) with an ABI QuantStudio 6 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Changes in the target genes were analyzed by the $2^{-\Delta\Delta Cq}$ method. We used the expression of the housekeeping gene 18S RNA as the reference for our analysis.

A total of 1.5×10^5 primary BMMs per well were inoculated in a 6-well plate and stimulated with RANKL (50 ng/mL) and metformin (0, 62.5, 125 μ mol/L) or alendronate (0, 62.5, 125 nmol/L), modulate osteoclast differentiation for 5 days. Then, total RNA extraction and subsequent experiments were performed as described above. The primer sequences are listed in Table 1.

Table 1 Primer sequences used in qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
Sox9	GAGCCGGATCTGAAGAGGGA	GCTTGACGTGTGGCTTGTTT
Aggrecan	CAGTGGGATGCAGGCTGGCT	CCTCCGGCACTCGTTGGCTG
ADAMTS5	GGAGCGAGGCCATTTACAAC	CGTAGACAAGGTAGCCCACTTT
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP13	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT
CTSK	CCAGTGGGAGCTATGGAAGA	AAGTGGTTCATGGCCAGTTC
DC-STAMP	TTCATCCAGCATTTGGGAGT	ACAGAAGAGAGCAGGGCAAC
AcP5	CAGCAGCCAAGGAGGACTAC	ACATAGCCCACACCGTTCTC
NFATC1	CAACGCCCTGACCACCGATAG	GGCTGCCTTCCGTCTCATAGT
c-Fos	TTTCAACGCCGACTACGAGG	GCGCAAAAGTCCTGTGTGTT
TNFRSF11	GAAGATGCTTTGGTGGGTGT	TCAGTCGGGATCAGTGTGAG
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

Western blotting

To evaluate the expression of AMPK signaling pathway factors during the anti-inflammatory effect of metformin, we inoculated ATDC5 cells in a 6-well plate. Then, we added IL-1 β to the wells after pretreating the cells with metformin (0, 5 mmol/L) for 2 h. Then, total protein was extracted from ATDC5 cells at 30 min using radioimmunoprecipitation assay (RIPA) lysis buffer (consisting of phosphatase inhibitors, and 100 mmol/l PMSF). To examine the expression of osteoarthritis-related proteins, ATDC5 cells were cultured in 6-well plates with various concentrations of metformin (0, 2, 4, and 5 mmol/L) with or without IL-1 β (10 ng/mL) for 5 days.

Similar to the real-time PCR assay, total protein was extracted from osteoclasts with radioimmunoprecipitation assay (RIPA) lysis buffer (consisting of phosphatase inhibitors, and 100 mmol/l PMSF). In addition, the signaling pathway-related proteins associated with osteoclast differentiation were analyzed at 0 min, 10 min, 20 min, 30 min, 60 min, and 120 min after 50 ng/mL RANKL was added to each well, which had been pretreated with metformin (0,125 μ M) for 2 h. The protein concentration was quantified using a BCA protein assay kit (BOSTER, Wuhan, China). Then, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to resolve the proteins. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and subsequently blocked with 5% nonfat milk for 2 h. The samples were then incubated overnight at 4 °C with the following primary antibodies: anti-SOX9 antibody (1:5000, Cat. #ab185966), anti-aggrecan (1:200,

Cat. #absc166951), anti-MMP3 (1:500, Cat. #AF0217), anti-MMP13 (1:500, Cat. #AF5355), anti-AMPK alpha 1 (1:200, Cat. #10929-2-AP), anti-phospho-AMPK alpha 1 (1:500, Cat. #AF3422), anti-cathepsin K (1:200, Cat. #sc48353), anti-NFATc1 (1:200, Cat. #sc7294), anti-c-Fos (1:500, Cat. #AF0132), anti-NF- κ B (anti-p65) (1:500, Cat. #BF0382), anti-phospho-NF- κ B (anti-pp65) (1:500, Cat. #AF2006). The membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBST) 15 min each time) and incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour. After being washed with TBST three times, the membranes underwent ECL development. We visualized the immunoreactive bands by measuring the gray values with ImageJ.

Histological and Immunohistochemical Analysis

The surgical side or normal joints from the mice were fixed in 4% paraformaldehyde and embedded in paraffin. Serial 6- μ m-thick sections were cut and collected on glass slides. Then, the samples were stained with hematoxylin and eosin (H&E) or safranin O/fast green (SO/FG). The degeneration of articular cartilage was scored by the Osteoarthritis Research Society International (OARSI) guidelines[19]. TRAP-positive cells attached to subchondral bone were stained with TRAP. For immunohistochemistry, 6- μ m sagittal sections were prepared from the paraffin-embedded tissue and treated with the following primary antibody anti-MMP13 (1:500, Cat. #AF5355), anti-phospho-AMPK alpha 1 (1:500, Cat. #AF3422), anti-Col II (1:100, Cat. #ab34712) overnight at 4 °C. Then, the cells were incubated with anti-rabbit secondary antibodies for 1 h at 37 °C and examined by DAB.

Immunofluorescence staining

ATDC5 cells were maintained in 6-well plates containing coverslips and stimulated with 5 mmol/L metformin with or without 10 ng/ml IL-1 β for 48 h. Then, the coverslips were fixed in 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 20 min. The coverslips were blocked with goat serum at 37 °C for 1 hour. Next, the coverslips were incubated with the following primary antibodies: anti-SOX9 antibody (1:100, Cat. #ab185966), and anti-MMP13 (1:500, Cat. #AF5355) overnight at 4 °C. After three washes with PBS, the cells were incubated with fluorescent secondary antibodies. DAPI was used for nuclear staining. Fluorescence inverted microscopy (CKX53; Olympus, Tokyo, Japan) was used to obtain immunofluorescent images.

Detection of reactive oxygen species (ROS) in vitro

For intracellular ROS analysis, Reactive oxygen species assay kit (Solarbio, Beijing, China) was used. ATDC5 cells were pretreated with 5 mmol/L metformin, incubated with 10 μ mol/L 2',7'-dichlorofluorescein diacetate (DCF-DA) for 20 minutes in the dark, and then washed three times with DMEM. Next, the cells were stimulated with IL-1 β for 5 min. ROS production was observed by fluorescence inverted microscopy. M-CSF-induced BMMs were seeded at a density of 1.5 $\times$ 10⁵/well in 6-well cell culture plates and stimulated with 50 ng/ml RANKL without or with 125 μ M metformin for 3 days. Then, the cells were incubated with 10 μ mol/L DCF-DA for 20 minutes in the dark, and ROS production was examined by Fluorescence inverted microscopy as described above.

Statistical analysis

All quantitative data from multiple experiments were calculated by GraphPad Prism 8 and are presented as the mean $\pm$ standard deviation (SD). The experiments were repeated three times. One-way analysis of variance was used to calculate the p value, and a p value of < 0.05 was considered to indicate a statistically significant result.

Results

Metformin inhibited IL-1 β -induced chondrocyte matrix catabolism in ATDC5 cells

It has been shown that IL-1 β can inhibit the metabolism of the chondrocyte matrix. In high-density culture experiments, treatment by IL-1 β resulted in a significant reduction in Alcian blue staining as assessed by the staining area multiplied by the grey value, whereas the metformin co-stimulation group had abundant Alcian blue staining, indicating that matrix degradation was significantly inhibited by 5 mmol/L metformin. On the other hand, there was no difference between the control and metformin-treated groups, regardless of the concentration of metformin (Fig. 1a, b). Immunofluorescence results also showed that 5 mmol/L metformin inhibited the IL-1 β -induced increased expression of MMP13 and decreased expression of SOX9 in ATDC5 cells (Fig. 1d). The CCK-8 assay demonstrated that 2, 4, and 5 mmol/L metformin had no significant effect on ATDC5 cell viability. When ATDC5 cells were treated with both IL-1 β and 2, 4, and 5 mmol/L metformin, the proliferation of ATDC5 cells was promoted at 48 h (Fig. 1c).

Real-time PCR showed that the expression of matrix metalloproteinase 3 (MMP3) and matrix metalloproteinase 13 (MMP13) was upregulated by IL-1 β . However, when cells were stimulated with both IL-1 β and metformin, the result was altered (Fig. 2a, 2b). Consistent with the real-time PCR results, western blot analysis showed that the expression of MMP13 and MMP3 was increased in ATDC5 cells treated with IL-1 β (Fig. 2e), while SRY-box transcription Factor 9 (SOX9) and aggrecan expression was decreased (Fig. 2e). Metformin decreased IL-1 β -induced MMP3 and MMP13 generation but enhanced sox9 and aggrecan expression, although Real-time PCR showed no statistical difference in sox9 and aggrecan expression (Fig. 2c, 2d). We suggest that this may be because protein degradation is inhibited by metformin. These data suggest that metformin plays an anti-inflammatory role in IL-1 β -induced ATDC5 cells.

Metformin protected against IL-1 β -driven catabolism in cartilage explants

To explore whether metformin could modulate extracellular matrix (ECM) generation during OA progression, “relatively normal” human cartilage explants were cultured in the presence or absence of metformin with or without IL-1 β for 24 h, 48 h, and 72 h. After being cultured, proteoglycan content, which was determined by SO/FG staining, significantly decreased in the IL-1 β treatment group compared with the control group, especially at 72 h. Moreover, the proteoglycan content was restored by 5 mmol/L metformin. Immunohistological staining showed that metformin enhanced the type II collagen (COL II), and reduced the MMP13 contents after 3 days of treatment (Fig. 3).

Metformin and alendronate inhibited RANKL-induced osteoclast differentiation

Before examining the effects of metformin and alendronate on osteoclast differentiation, we tested the cytotoxicity of these drugs on BMMs using a CCK-8 assay. The results demonstrated that metformin did not inhibit cell proliferation at concentrations of 0-250 $\mu\text{mol/L}$ (Fig. 4a). Then, BMMs were incubated with metformin and stimulated with M-CSF plus RANKL. TRAP staining was used to evaluate reactive osteoclast formation. TRAP-positive cells containing more than three nuclei were considered osteoclasts (Fig. 4b, 4c). Our results indicated that BMMs incubated with metformin (62.5 $\mu\text{mol/L}$, 125 $\mu\text{mol/L}$) had fewer TRAP-positive cells than BMMs incubated without metformin. This result revealed that metformin had an inhibitory effect on osteoclast differentiation. We also found that osteoclast formation was lower in groups treated with 125 nmol/L and 62.5 nmol/L alendronate than in the control group, indicating that alendronate inhibited osteoclast differentiation (Fig. 4b, 4c). Moreover, the CCK-8 assay showed no significant difference in the viability of BMMs after treatment with different concentrations of alendronate, suggesting that alendronate has no toxic effect on living cells at concentrations of 0-125 nmol/L. (Fig. 4a).

Metformin and alendronate downregulated the expression of osteoclast differentiation-related genes

Because cellular oncogene FOS (c-Fos), dendritic cell-specific transmembrane protein (DC-STAMP), TNFRSF11, and cathepsin K (CTSK) are critical genes associated with RANKL-induced osteoclast differentiation, we examined the effects of metformin and alendronate on osteoclast differentiation at the genetic level by real-time PCR. The results showed that osteoclast function-related genes, such as CTSK, were upregulated by RANKL treatment (Fig. 3a), and DC-STAMP, which is associated with osteoclast fusion, was similarly upregulated (Fig. 3a). Metformin and alendronate together decreased the RANKL-induced mRNA expression of target genes (Fig. 3a). TNFRSF11 expression was enhanced by RANKL, and this gene encodes the RANKL protein. Our results showed that the expression of the TNFRSF11 gene was downregulated in osteoclasts in the metformin-treated and alendronate-treated groups (Fig. 3a). These data confirm that metformin and alendronate both have inhibitory effects on RANKL-induced osteoclast formation and osteoclast function.

NFATc1 expression was inhibited by metformin and alendronate

The nuclear factor of activated T cells c1 (NFATc1) induction is a hallmark event in determining the fate of osteoclasts, and NFATc1 activation induces the transcription of osteoclast-specific genes such as CTSK[20]. We measured the expression of NFATc1, and CTSK by western blotting. We found that metformin inhibited the expression of these two proteins compared to that in cells treated with RANKL alone (Fig. 3b, 3c). we investigated the expression of c-Fos, the key transcription factors in osteoclastogenesis. Metformin inhibited the RANKL-induced c-Fos generation (Fig. 3a, 3b, 3c). This suppression was also induced by alendronate (Fig.3a, 3b, 3c).

Metformin inhibited inflammation and osteoclast differentiation via the AMPK pathway, NF- κ B pathway

As metformin is a known AMPK activator[21], we examined whether metformin upregulated the expression and phosphorylation of AMPK α 1 in IL-1 β -treated ATDC5 cells using western blotting. IL-1 β significantly reduced the levels of phosphorylated AMPK α 1, while metformin (5 mmol/L) reversed the effect on AMPK α 1 phosphorylation at 2 h (Fig. 6a). Moreover, the addition of metformin reversed the inflammatory response caused by IL-1 β in the ATDC5 cell line. These results were confirmed in the mouse knee joint by immunohistochemistry. The expression of phosphorylated AMPK α 1 in the articular chondrocytes of knee cartilage was decreased after DMM surgery, and metformin enhanced AMPK α 1 phosphorylation in articular chondrocytes after DMM surgery (Fig. 8a, 8d). During RANKL-induced osteoclastogenesis, the decrease in p-AMPK levels was reversed by 125 μ mol/L metformin at 10 min, 20 min, 30 min, 60 min, and 120 min. Moreover, nuclear factor- κ B (NF- κ B) activation, which is a key molecular event in early RANKL-induced osteoclastogenesis, was significantly inhibited by metformin in the presence of RANKL at 20 min, 30 min, 60 min, and 120 min (Fig. 6f).

Metformin inhibited oxidative stress in ATDC5 cells and BMMs

Because oxidative stress has been linked to the pathogenesis of osteoarthritis due to its role in chondrocyte apoptosis and extracellular matrix (ECM) degradation[22], we determined whether metformin could suppress IL-1 β -induced oxidative stress in ATDC5 cells by measuring ROS production. We found that pretreatment with metformin (5 mmol/L) significantly inhibited IL-1 β -mediated production of ROS in OA chondrocytes (Fig.6b). Osteoclast formation and activity are induced by elevated ROS. In our study, ROS levels in BMMs treated with metformin plus RANKL were significantly lower than those in RANKL-treated BMMs and were comparable to basal levels in untreated BMMs (Fig.6k). Therefore, these data indicate that metformin may possess antioxidant properties.

Combined treatment with metformin and alendronate limited OA development in the DMM mouse model through chondroprotective effects and inhibiting subchondral bone remodeling

The DMM mouse model was used to validate the protective effects of alendronate plus metformin on OA development. The overall structure of the knee joint and the loss of cartilage were examined by H&E, SO, and immunohistochemistry staining. As expected, slightly stripped cartilage and less safranin O staining were observed in the DMM group (Fig.7a). However, the amount of cartilage damage in the alendronate-treated group was lower than that in the DMM group. Moreover, less cartilage damage was observed in the combined alendronate and metformin treatment group than in the other groups (Fig. 7a). The OARSI score in the combined alendronate and metformin treatment group was significantly lower than that in the DMM group (Fig. 7b). Similarly, we used immunohistochemistry to show that the expression of MMP13 was dramatically increased in the DMM group, and the significantly increased expression of MMP13 in cartilage was attenuated by combined treatment with alendronate and metformin. But, the expression of COL II was enhanced (Fig.8a, 8b, 8c). We also measured the number of osteoclasts in the subchondral bone by TRAP staining. After surgery, the number of osteoclasts was increased in the subchondral bone in the DMM group. This effect was abrogated by alendronate, and this preventive effect was further enhanced by combined treatment with alendronate and metformin (Fig. 7a, 7c).

Discussion

Alendronate, a bisphosphonate, is a bone turnover inhibitor that is used to treat osteoarthritis due to its ability to protect chondrocytes by inhibiting matrix metalloproteinase expression and subchondral bone reconstruction [23]. A trial to evaluate bisphosphonates as structural modifiers in osteoarthritis nears completion. The future use of this therapy is trending to less frequent administration because of the potential for excessive inhibition of conversion with prolonged use, and interactions with anabolic therapies are currently being examined. In the present study, we found that alendronate combined with metformin delayed the progression of OA in mice by inhibiting inflammation and attenuating RANKL-induced bone resorption. Mechanistically, metformin suppressed osteoclastogenesis and improved cartilage matrix synthesis by reducing ROS production and mediating AMPK signaling, and NF- κ B signaling.

IL-1 β was used to establish an invitro inflammation model in ATDC5 chondrocytes and induced cartilage matrix degradation, which was shown by Alcian blue staining. Features of osteoarthritis include degradation of the extracellular matrix (ECM) and loss of cartilage gene expression in articular cartilage. As expected, in response to IL-1 β , the expression of aggrecan and SOX9 was decreased, while matrix metalloproteinases (MMP3 and MMP13), which are enzymes that can degrade all components of the ECM, were upregulated in the ATDC5 chondrocytes. MMP13 is a substrate-specific enzyme whose overexpression can lead to cartilage degeneration, as it degrades the proteoglycans aggrecan and collagen, both of which are components of articular cartilage[24]. The expression of MMP3 is increased in arthritis, and this protein mainly degrades non-collagenous matrix components of the joint. In our study, we found that the upregulation of MMP3 and MMP13 expression by IL-1 β was eliminated by metformin. SOX9, a master regulator of Aggrecan and collagen, plays roles in both cartilage development and homeostasis by directly targeting genes encoding cartilage-specific extracellular matrix components[25]. Therefore, modifying the level of Sox9 expression in OA cartilage may be beneficial in improving the course of OA. Our results showed that metformin upregulated SOX9 expression, which was inhibited by IL-1 β . Aggrecan is one of the main structures in the extracellular matrix of articular cartilage, and it is well known that the loss of Aggrecan accelerates the progression of osteoarthritis[26]. As we hypothesized, metformin abrogated the inhibitory effect of IL-1 β on Aggrecan. Consistent with the results of cellular experiments, treatment with inflammatory cytokines (IL-1 β) caused cartilage tissue degradation. Metformin treatment reduced the degree of S/F staining loss in human articular cartilage explants cultured in the presence of IL-1 β . Furthermore, metabolism in the cartilage explants remained stable for up to 72 h, and there was no significant difference in the proteoglycan content in the absence of IL-1 β at 24, 48, and 72 h. These results suggest that metformin may play a protective role in OA.

Currently, numerous studies suggest that changes in the microarchitecture of subchondral bone precede articular cartilage degradation in OA[13]. Early-stage increased bone loss in subchondral bone is an important component of the pathogenetic process that leads to OA[13]. In our experimental model, DMM-OA mice treated with alendronate plus metformin exhibited reduced numbers of TRAP⁺ multinucleated osteoclasts and articular cartilage degeneration with improved OARSI scores compared to DMM-OA mice.

This is the result of the inhibition of osteoclast formation and bone resorption by alendronate in combination with metformin. There was a downward trend between the alendronate-treated and untreated groups but no significant difference. This outcome may be the result of the low-frequency effect of low concentrations of alendronate. We also found that both metformin and alendronate inhibited RANKL-induced osteoclast formation in vitro. In this study, RANKL-induced osteoclasts were confirmed by TRAP staining, and the expression of osteoclast marker genes and proteins, including c-Fos, NFATc1, CTSK, and DC-STAMP, was quantitatively examined by western blotting and RT-PCR. CTSK, which is specifically expressed by osteoclasts[27], is essential for osteoclast-mediated bone resorption[28]. NFATc1, which is the transcription factor that is most potently induced by RANKL, works together with other transcription factors to induce various osteoclast-specific genes[29]. The TRAP, calcitonin receptor, and cathepsin K genes, all of which are important for osteoclast function, are regulated by NFATc1. DC-STAMP, which was shown to be essential for the cell-cell fusion of osteoclast precursor cells, has been shown to be a transcriptional target of NFATc1. The c-Fos, which is an indispensable downstream regulatory factor of RANKL, accelerates the formation of osteoclasts by activating the downstream factor NFATc1[30]. In this study, both metformin and alendronate inhibited the expression of these factors. Thus, metformin and alendronate may inhibit osteoclast formation by downregulating the expression of these genes.

We further investigated the mechanism by which metformin inhibits inflammation and osteoclasts in osteoarthritis. AMP-activated protein kinase (AMPK) is a key molecule in the antidiabetic mechanism of metformin and is also involved in the signaling pathways associated with bone physiology. AMPK has three subunits (α , β , γ), and its catalytic activity is associated with the α subunit[31]. Phosphorylation of Ser486 in the α subunit triggers AMPK activation. In OA human articular cartilage, decreased AMPK α phosphorylation was observed compared to that in the normal cartilage group[32]. In our study, the protein expression of p-AMPK α 1 was significantly inhibited by IL-1 β . This inhibition, as well as MMP expression, was reversed after treatment with metformin, and these results suggest that the anti-inflammatory effects of metformin involve the AMPK signaling pathway. Conversely, studies have shown a negative regulatory effect of AMPK on cartilage differentiation[8]. In line with this, our experiments showed that the expression of SOX9, a marker gene of cartilage differentiation, was repressed in the presence of metformin. Aggrecan expression was also suppressed. This finding suggests that AMPK activity may have a protective effect on the degradation of articular cartilage. Moreover, impairments in AMPK contribute to mitochondrial dysfunction. ROS, which is increased in response to mitochondrial dysfunction, promotes cartilage degradation in OA by breaking down collagen, aggregating proteoglycans, and activating matrix metalloproteinases (MMPs)[33] and is also connected to AMPK signaling. Our in vitro studies showed an increase in ROS in IL-1 β -treated inflammatory chondrocytes. In the presence of metformin, phosphorylated AMPK α 1 abrogated the increased ROS level. Our data reveal that metformin-induced reductions in ROS production and AMPK phosphorylation may be protective mechanisms in chondrocytes. AMPK is a negative regulator of RANKL. The binding of RANKL to its receptor RANK leads to the activation of the c-Fos signaling pathway, which activates NFATc1, a transcription factor associated with osteoclast differentiation, fusion, and activation[34]. Evidence has

shown that RANKL induces the phosphorylation/activation of AMPK- α in BMMs and stimulates the formation of TRAP-positive multinucleated cells[35]. There are two different α isoforms, α 1, and α 2, and the α 1-isomer is mainly expressed in BMMs. We hypothesized that metformin could inhibit RANKL-induced osteoclast formation in the presence of M-CSF by activating/phosphorylating AMPK α 1. Consistently, in our study, NFATc1 and c-Fos were significantly reduced by metformin after treatment with RANKL, while metformin-induced AMPK α 1 phosphorylation was maintained. Thus, metformin plays an important role as a negative regulator of RANKL-mediated osteoclastogenesis by acting as an inhibitor of NFATc1 activation through c-Fos and AMPK signaling. It has also been shown that the increase in ROS in BMMs can be attenuated by metformin[36]. ROS can be involved in osteoclast formation through various signaling pathways, including the MAPK, PI3K, and NF- κ B pathways[37]. In our study, metformin pretreatment reduced the RANKL-induced increase in ROS levels in BMMs. This finding suggests that the regulation of intracellular ROS levels may be a target by which metformin inhibits osteoclast differentiation. Regardless, osteoarthritis is considered a whole-joint disease that involves both chondrocytes and osteoclasts, and metformin-mediated targeting ROS and inhibiting inflammation through AMPK activation will greatly slow the progression of osteoarthritis. We further showed that metformin regulated the NF- κ B signaling pathway. Persistently elevated responses in the NF- κ B pathway are associated with diseases such as rheumatoid arthritis (RA) and OA[38]. Activation of the transcription factor NF- κ B in turn activates genes that regulate osteoclast differentiation[39]. In our study, metformin inhibited RANKL-induced NF- κ B phosphorylation in osteoclasts. Although it remains unclear how metformin-mediated AMPK signaling affects NF- κ B, we suspect that metformin exerts a therapeutic effect on OA through the AMPK and NF- κ B pathways.

Alendronate similarly inhibited osteoclast differentiation and bone resorption. We found that alendronate downregulated the expression of c-Fos and NFATc1, suggesting that alendronate may inhibit the damaging effects of OCs on subchondral bone by interfering with the c-Fos-NFATc1 pathway.

Conclusion

In conclusion, metformin combined with alendronate inhibited inflammatory factors and osteoclast differentiation, improved cartilage matrix metabolism and subchondral bone reconstruction, and delayed the progression of OA in mice. Metformin combined with alendronate may be a useful treatment for attenuating early-stage OA. This would be an ideal drug/drug combination for treating osteoarthritis in the presence of osteoporosis combined with T2D. In addition, this approach needs to be further validated in a clinical setting.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Yanjing Guo and Shaowei Wang contributed to conception. Yanjing Guo, Chunfang Wang, and Shaowei Wang designed the study, and Yanjing Guo wrote the first draft of the manuscript. Sujing Zong, Zhenyu Wang, and Jiyu Zhao organized the database. Ruijia Yang, Chunfang Wang, and Chongwei Chen performed the statistical analysis. Ruijia Yang, and Chongwei Chen wrote sections of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of Shanxi Medical University (2018LL039).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

Figure 1

Metformin enhanced anabolism and inhibited IL-1 β -induced chondrocyte matrix catabolism in ATDC5 cells and is not cytotoxic in vitro. (a) Alcian blue staining was quantified. (b) Alcian blue staining of ATDC5 cells. (c) Effects of metformin on ATDC5 cells viability by the CCK-8 assay. N=3. *P < 0.05, ***P < 0.001, ****P < 0.0001 vs. control group (without IL-1 β induction and metformin treatment). (d) Immunofluorescence analyses were used to determine the protein level of sox9 and MMP13 in ATDC5 cells. Scale bars, 20 μ m. N=3. Data are presented as mean $\pm$ standard deviation of the mean (SD). *P < 0.05, **P < 0.01, ****P < 0.0001 vs. IL-1 β group. Statistical analysis was conducted using a one-way analysis of variance.

Figure 2

Metformin alleviates IL-1 β -induced degradation of the extracellular matrix. (a - d) The expression level of (a) MMP13, (b) MMP3, (c) Aggrecan, and (d) SOX9 mRNA in ATDC5 cells were determined by qRT-PCR. (e) The expression level of Aggrecan, Sox9, MMP3, and MMP13 protein in ATDC5 cells were determined by western blot. (f - i) Quantitative analysis of the differential expression level of (f) Aggrecan, (g) SOX9, (h) MMP3, and (i) MMP13. The GAPDH was selected as the control protein. N=3. Data are presented as mean $\pm$ standard deviation of the mean (SD). *P < 0.05, **P < 0.01, ****P < 0.0001 vs. IL-1 β group. Statistical analysis was conducted using one-way analysis of variance.

Figure 3

Metformin protected against IL-1 β -driven ECM degradation in cartilage explants. The proteoglycan content of cartilage explants was measured by safranin O-fast green staining after 24 h, 48 h, and 72 h of incubation with metformin or metformin + IL-1 β . Immunohistochemistry staining of COLII and MMP13 in control, IL-1 β , metformin, metformin + IL-1 β group after 72 h (safranin O-fast green stain, scale bars, 100 μ m. COLII, MMP13, scale bars, 50 μ m). N=3.

Figure 4

Metformin and alendronate inhibit in vitro osteoclastogenesis. (a) Effects of metformin and alendronate on BMMs viability by the CCK-8 assay. (b) Quantification analysis of TRAP-positive multinucleated cells (MNCs, nuclei ≥ 3). (c) TRAP staining shows the numbers of TRAP-positive cells (>3 nuclei) in BMMs treated with 50 ng/mL M-CSF, 50 ng/mL RANKL, and metformin or alendronate. N=3. Data are presented as mean $\pm$ standard deviation of the mean (SD), ****P < 0.0001 vs. RANKL group. Statistical analysis was conducted using one-way analysis of variance.

Figure 5

Metformin and alendronate inhibit in vitro osteoclastogenesis and suppresses osteoclastogenesis-related gene and protein expression. (a) qRT-PCR analysis of the mRNA levels of osteoclastogenesis-related genes, c-Fos, CTSK, DC-STAMP, and TNFRSF11. The 18sRNA was selected as the control gene. (b) Quantification of the ratios of band intensity of NFATc1, c-Fos, and CTSK relative to GAPDH. (c) Western blotting was used to determine the protein level of NFATc1, c-Fos, and CTSK. N=3. Data are presented as mean $\pm$ standard deviation of the mean (SD), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. RANKL group. Statistical analysis was conducted using one-way analysis of variance.

Figure 6

Metformin reduced oxidative stress as well as inhibited inflammation and osteoclast differentiation via the AMPK signaling pathway, and NF- κ B signaling pathway. (a) Western blot assay was performed to detect the protein expression level of AMPK α 1, and p-AMPK α 1 in ATDC5 cells. (b) Representative confocal images of IL-1 β -induced ROS production in ATDC5 cells in the presence or absence of pre-treated metformin. (c - d) Quantification of the ratios of band intensity of AMPK α 1, and p-AMPK α 1 relative to GAPDH. (e) The ratios of band intensity of p-AMPK α 1 / The ratios of band intensity of AMPK α 1. N=3, *P < 0.05 vs. IL-1 β group. (f) Western blot images show the levels of AMPK α 1, p-AMPK α 1, NF- κ B-p65, and p-NF- κ B-p65 at 0, 10, 20, 30, 60, and 120 mins in the RANKL- and RANKL + metformin-treated BMMs. (g - j) Quantification of the ratios of band intensity of (g) AMPK α 1, (h) p-AMPK α 1, (i) NF- κ B-p65, and (j) p-NF- κ B-p65 relative to GAPDH. (k) Representative confocal images of RANKL-induced ROS production in BMMs with and without pre-treated of metformin. N=3. Data are presented as mean $\pm$ standard deviation of the mean (SD), *P < 0.05, **P < 0.01, ****P < 0.0001 vs. RANKL group. Statistical analysis was conducted using one-way analysis of variance.

Figure 7

Combined treatment with metformin and alendronate limited OA development in the DMM mouse model through chondroprotective effects and inhibiting subchondral bone remodeling. (a) Representative images of H&E, safranin O, TRAP staining in DMM, alendronate-treated, alendronate + metformin-treated mice after 8 w of administration. (b) The corresponding Osteoarthritis Research Society International (OARSI) scores from DMM, alendronate-treated, and alendronate + metformin-treated mice. (c)

Quantitative measurements of Oc.S / BS in the no-treatment group, alendronate-treated group, and alendronate + metformin-treated group. (d -f) The number of COLII, MMP13, and p-AMPK α 1 positive cells in the knee cartilage. (j) Immunohistochemistry staining of COLII, MMP13, p-AMPK α 1 in DMM, alendronate-treated, alendronate + metformin-treated mice after 8 w of administration. TRAP staining, scale bar, 100 μ m; H&E, safranin O staining, scale bar, 50 μ m. N=5. Data are presented as mean $\pm$ standard deviation of the mean (SD), *P < 0.05, **P < 0.01, ****P < 0.0001 vs. DMM group. Statistical analysis was conducted using one-way analysis of variance.

Figure 8

Combined treatment with metformin and alendronate limited OA development and induced phosphorylation and expression of AMP-activated protein kinase (AMPK) in the DMM mouse model. Immunohistochemistry staining of COLII, MMP13, p-AMPK α 1 in DMM, alendronate-treated, alendronate + metformin-treated mice after 8 w of administration. (a) Immunohistochemistry staining, scale bar, 50 μ m (upper panel) and 20 μ m (lower panel). (b-d) The number of (b) COLII, (c) MMP13, and (d) p-AMPK α 1 positive cells in the knee cartilage. N=5. Data are presented as mean $\pm$ standard deviation of the mean (SD), *P < 0.05, **P < 0.01, ****P < 0.0001 vs. DMM group. Statistical analysis was conducted using one-way analysis of variance.

Figure 9

Mechanisms by which metformin and alendronate limit the progression of osteoarthritis by targeting chondrocytes and osteoclasts.