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Original Article

Alendronate impact on Col II, MMP-13, and β-catenin in osteoarthritis rats

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Abstract: As one of the common osteoarticular disease, osteoarthritis (OA) presents pathological changes including osteocyte damage, new bone formation, bone trabecula fibrotic sclerosis, and bone neoplasm. As the third-generation bisphosphonates, Alendronate (ALN) has strong affinity to hydroxyapatite in bone. It can inhibit reabsorption to prevent bone remodeling and accelerate bone resorption activity. We intended to provide experimental basis of ALN in treating OA. Knee cartilage was extracted from 30 male SD rats. The chondrocytes were passaged to the third generation through culture in vitro. The chondrocytes were divided into blank group, IL-1β induction group, and ALN intervention group. Col II, MMP-13, and β-catenin expression were detected. Compared with IL-1β induction group, ALN intervention can upregulate Col II expression significantly ($P < 0.01$). Compared with IL-1β induction group, ALN intervention can increase β-catenin expression obviously ($P < 0.01$). Compared with IL-1β induction group, ALN intervention can decline MMP-13 expression markedly ($P < 0.01$). ALN upregulates Col II, MMP-13, and β-catenin expression in chondrocytes from IL-1β induced OA rats and protects chondrocytes growth.

Keywords: OA, ALN, Col II, MMP-13, β-catenin

Introduction

As one of the common osteoarticular diseases, osteoarthritis (OA) is mainly in the elderly population. There are more female patients than male. Its main clinical manifestation includes large joint pain, inflexible joint activity, bad flexion degree, and even ankylosis. It seriously affects quality of life. OA patients gradually increase along with our country entering aging society, bringing large burden to the society. As a type of joint injury disease, it mainly features as focal articular cartilage degeneration, bone loss, joint marginal osteophyte formation, joint deformity, and subchondral bone hardening. The pathological changes include osteocytes damage, new bone formation, bone trabecular fibrotic sclerosis, and bone neoplasm formation. Its major pathological manifestations are articular cartilage degeneration, cartilage surface fibrosis, matrix proteoglycan loss, collagen matrix damage, subchondral bone exposure, and osteophyte formation. Its pathological damage factors include cell dysfunction, cell apoptosis, osteocytes dysfunction, apoptosis, inflammatory factors, and degrading protease secretion increase [1, 2]. Alendronate (ALN) is a representative drug of diphosphate that has strong affinity with hydroxyapatite in bone. It can enter the hydroxyapatite crystals in bone matrix and is released to inhibit osteoclast activity when osteoclast dissolved the crystals. It is featured as suppressing reabsorption that prevents bone remodeling, accelerates antiresorption activity, and inhibits no bone mineralization [3]. This study extracted cartilage cells in knee from IL-1β induced rats in vitro and tested Col II, MMP-13, and β-catenin expression after ALN intervention. We aimed to provide experimental basis for ALN treatment on OA by investigating ALN influence on OA chondrocytes.

Materials and methods

Animals

100 healthy male SD rats in clean grade were provided by Southern Medical University. The

Table 1. Real time PCR primer sequence

Gene	Primer sequence	Length (bp)
Col II	F 5'-GTGGAGCAAGAGCAAGGA-3'	334
	R 5'-CTTGCCCCACTTACCAGTGTG-3'	
MMP-13	F 5'-TGACTATGCGTGGCTGG-3'	866
	R 5'-GGGAAGGGGCTAATGAACA-3'	
β-catenin	F 5'-TGCAGCGACTAAGCAGGA-3'	198
	R 5'-TCACCAGCACGAAGGACA-3'	
β-actin	F 5'-CAAGGGCCAGGTCACCAA-3'	140
	R 5'-CCCCCAACCCATCTTCGT-3'	

average weight was (220.34 ± 12.32) g. The temperature and relative humidity of the lab was 21-26°C and 50~60%, respectively. Experiments on animals conformed to animal ethics standard.

Reagents and instruments

ALN was purchased from Shijiazhuang Haisenhuagong Co., LTD. Col II, MMP-13, and β-catenin polyclonal antibodies were got from Beijing Zhongshan Biotechnology Co., LTD. Recombinant human IL-1β was bought from Shanghai Colette Biotechnology Company. Real time PCR MasterMix was from Northwestern University Medical Engineering Department. Motic 6.0 medical image acquisition and analysis system was purchased from Gloud.

Chondrocytes cultivation

30 male SD rats were terminated by spinal dislocation and soaked in 75% ethanol for 10 min. Bilateral hindlimbs were isolated and the knee joint surface was cultured in blank medium with streptomycin.

The knee joint of hindlimb was separated to knee joint cartilage surface excluding the synovial membrane on the cartilage surface. Then the cartilage was isolated as 1 mm × 1 mm tissue and put into centrifugal tube containing 1.5 mL 0.2% type II collagenase after washed by PBS for three times. After incubated in 37°C water bath for 4 h and centrifuged for 5 min, the supernatant was removed. Osteocytes were cultured 25 cm^2 flask with complete DMEM medium (15% FBS and 100 U/ml streptomycin) at 2 × 10^5/ml and maintained in 37°C and 5% CO_2 for 72 h. Then the cells medium was changed every other day and passaged. The osteocytes in the third generation were used for experiment [4].

Grouping

The third-generation cartilage cells were randomly divided into three groups, including control group (n = 10), IL-1β induction group (n = 10), and ALN intervention group (n = 10). The cartilage cells in control were cultured in complete DMEM medium for 7 d. The cartilage cells in IL-1β induction group were added with 10 ng/ml recombinant human IL-1β for two days from the second day. The cartilage cells in ALN intervention group were treated with 10 ng/ml recombinant human IL-1β for two days from the second day, and then cultured in ALN medium at 1 × 10^{-6} mol/L for the left five days.

Immunocytochemistry

At first, the cartilage cells were soaked in 4% paraformaldehyde for 40 min and washed by PBS for three times. Then the cells were incubated in 3% H_2O_2 under room temperature for 10 min and blocked with 50 μl 5% blocking buffer for 20 min. Next, the cells were incubated in 50 μl Col II, MMP-13 or β-catenin polyclonal antibody at 4°C overnight. PB instead of primary antibody was used as negative control. At last, the cells were incubated with secondary antibody according to the manual and analyzed by Image Pro Plus 6.0 analysis software. Integral absorbance (IA) value of the Col II, MMP-13 or β-catenin was detected. Stronger positive expression and tan particles increase in cells corresponded to higher IA value.

PCR detection

Total RNA was extracted using Trizol and reverse transcripted to cDNA. PCR reaction was applied on Roter-Gene 3000 real time PCR system, and the primers were listed in **Table 1**. Ct value of Col II, MMP-13, and β-catenin were calculated based on control group and reference gene.

Statistical analysis

SPSS 17.0 software was used for data analysis. Measurement data was presented as mean ± standard deviation. ANOVA or SNK test were used for comparison. $P < 0.05$ was considered with statistical significance.

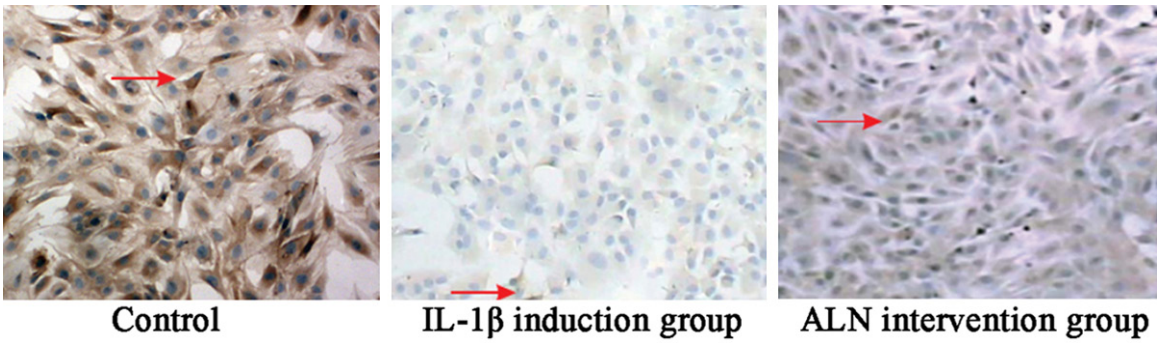

Figure 1. Col II immunocytochemistry observation (× 100), arrowhead means positive cell.

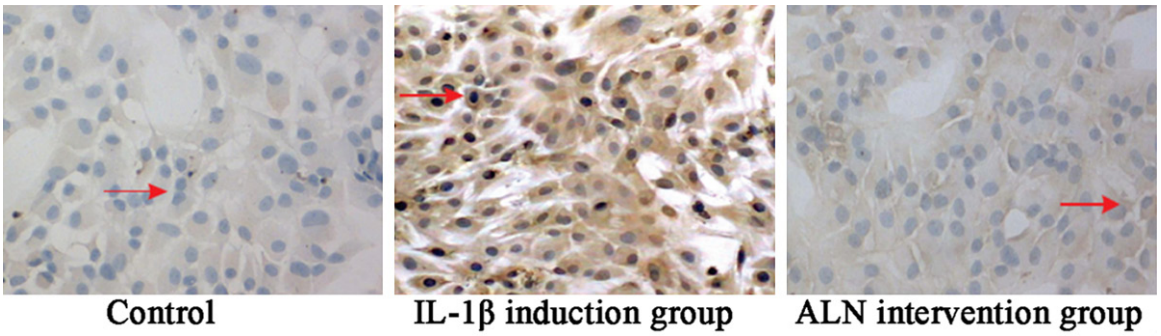

Figure 2. MMP-13 immunocytochemistry observation (× 100), arrowhead means positive cell.

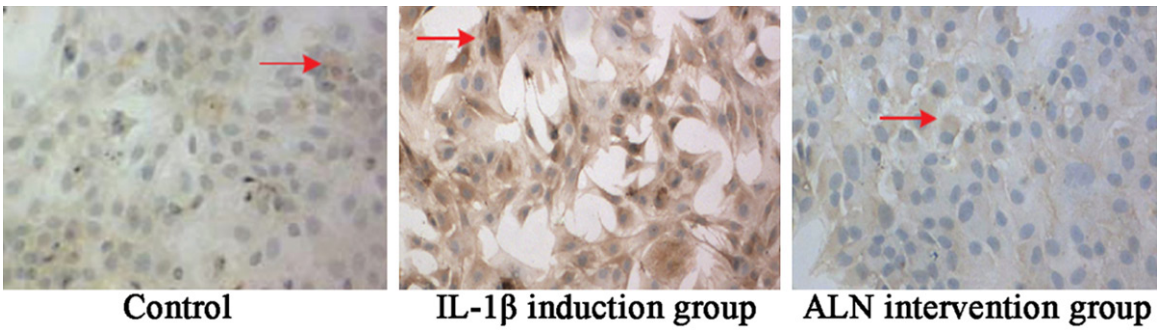

Figure 3. β-catenin immunocytochemistry observation (× 100), arrowhead means positive cell.

Results

Immunocytochemistry analysis

Col II: Cartilage cells in control showed strong positive as tan particles in cells. Cartilage cells in IL-1β induction group presented weak positive. Cartilage cells in ALN intervention group showed stronger positive than that in IL-1β induction group but weaker than in control (**Figure 1**).

MMP-13: Cartilage cells in control showed weak positive, while the cartilage cells in IL-1β induction group presented strong positive. Cartilage cells in ALN intervention group showed obviously weaker positive than that in IL-1β induction group but stronger than in control (**Figure 2**).

β-catenin: Cartilage cells in control showed weak positive, whereas the cartilage cells in IL-1β induction group showed strong positive. Cartilage cells in ALN intervention group showed significantly weaker positive than that in IL-1β induction group but stronger than in control (**Figure 3**).

Table 2. IA value of Col II, MMP-13, and β-catenin comparison (n = 10, χ ± s)

Group	Col II	MMP-13	β-catenin
Control	16..211 ± 1.241	2874 ± 0.178	5.140 ± 0.574
IL-1β induction	5.543 ± 0.578*	7.014 ± 0.471*	12.247 ± 0.987*
ALN intervention	10.574 ± 0.974*,Δ	3.102 ± 0.212*	8.658 ± 0.389*,Δ
F	187.141	61.247	159.217
P	0.0000	0.0000	0.0000

*P<0.01, compared with control; ΔP < 0.01, compared with IL-1β induction group.

Table 3. Ct value of Col II, MMP-13, and β-catenin comparison (n = 10, χ ± s)

Group	Col II	MMP-13	β-catenin
Control	1.0000 ± 0.0000	1.0000 ± 0.0000	1.0000 ± 0.0000
IL-1β induction	0.0795 ± 0.0421*	2.2471 ± 0.5471*	11.1027 ± 0.6897*
ALN intervention	3.2872 ± 0.5873*,Δ	0.0517 ± 0.0317*,Δ	4.1021 ± 0.8297*,Δ
F	147.274	66.274	283.247
P	0.0000	0.0000	0.0000

*P < 0.01, compared with control; ΔP < 0.01, compared with IL-1β induction group.

IA value of Col II, MMP-13, and β-catenin analysis

Col II: IA value in IL-1β induction group was significantly lower than that in control (P < 0.01). IA value in ALN intervention group was obviously higher than that in IL-1β induction group (P < 0.01).

Mmp-13: IA value in ALN intervention group was markedly lower than that in IL-1β group but higher than that in control (P < 0.01).

β-catenin: IA value in ALN intervention group reduced obviously compared with IL-1β induction group, while significantly increased compared with control (P < 0.01) (**Table 2**).

Col II, MMP-13, and β-catenin PCR comparison

Col II: Ct value in IL-1β induction group was significantly lower than that in control (P < 0.01). Ct value in ALN intervention group was obviously higher than that in IL-1β induction group (P < 0.01).

MMP-13: Ct value in ALN intervention group was markedly lower than that in IL-1β group but higher than that in control (P < 0.01).

β-catenin: Ct value in ALN intervention group reduced obviously compared with IL-1β induction group, while significantly increased compared with control (P < 0.01) (**Table 3**).

Discussion

The pathological characteristic of OA is chondrocytes morphology and function changes in articular cartilage [5]. In recent years, study found that inflammation participated in OA development process as an important role. IL-1β inhibited Col II secretion and promoted MMPs secretion in chondrocytes [6, 7]. According to previous reports, it was found that 10 ng/mL recombinant human IL-1β can induce healthy knee joint cartilage cells appeared OA changes that destroy chondrocytes to establish OA model [8].

As the representative third-generation bisphosphonates drug, ALN can delay OA cartilage decomposition [9]. Animal experiment showed that ALN subcutaneous injection can inhibit cartilage matrix degradation in the New Zealand white rabbit knee joint cartilage damage model [10]. The main clinical effect of ALN was to enhance the patient's physical activity, slow down articular cartilage degradation, and improve the quality of life [11]. In this study, ALN treatment dose was determined according to the Van Offel's result [12]. It was showed that Col II was the main part of the articular cartilage matrix that mainly responsible for maintaining cartilage normal development, and bisphosphonates can prevent Col II further degradation [13]. Our results revealed that ALN elevated Col II expression in OA chondrocytes, suggesting that ALN can enhance Col II gene and protein levels in cartilage cells. The study found that MMP-13 was the main catabolic enzyme of Col II, and ALN can reduce a variety of MMPs activity including MMP-13 and inhibit MMP-13 expression in cartilage [14]. Clinical study reported that ALN can effectively inhibit MMP-13 activity in rheumatoid arthritis [15]. In our study, MMP-13 expression in ALN intervention group was lower than that in IL-1β induc-

tion group, indicating that ALN can slow down MMP-13 expression and reduce Col II degradation in OA chondrocytes to repair cartilage cells. Study discovered that Wnt/β-catenin signaling pathway participated in OA pathological changes [16]. β-catenin mediated protein overexpression in the Wnt/β-catenin signaling pathway can damage the mature articular cartilage [17]. IL-1β induction can elevate β-catenin expression in chondrocytes cultured in vitro [18]. Experiments proved that β-catenin activation in cartilage cells can lead to OA changes [19, 20]. In this study, β-catenin expression improved in IL-1β induction group compared with control, suggesting that its elevation was one of the pathological changes of OA. β-catenin expression in ALN intervention group obviously decreased compared with IL-1β induction group, revealing that ALN can protect OA chondrocytes through intervening β-catenin expression.

To sum up, ALN can protect chondrocytes by regulating Col II, MMP-13, and β-catenin expression.

Disclosure of conflict of interest

None.

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