Chondro-protective Effects of Bisphosphonate for PTOA Could Attribute to the Inhibition of Chondrocyte Mevalonate Pathway

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Introduction: Zoledronic acid (ZA) is an FDA approved drug treatment for resorption-related bone loss. As a member in the bisphosphonate family, ZA deactivates osteoclasts by inhibiting the mevalonate pathway[1]. Our previous studies found that systematic injection of ZA can also suppress the development of post-traumatic osteoarthritis (PTOA) in the DMM (destabilization of medial meniscus) mouse model[2]. However, it is still unclear about how bisphosphonate could grant chondrocytes a new lease of life after traumatic damage. The central hypothesis of this study is that the chondro-protective function of ZA is attributable to its direct effect on chondrocytes. In specific, we will test the following hypothesis: 1) ZA can rescue the cartilage from traumatic damage during in vitro culture without the presence of subchondral bone, 2) ZA can promote the beneficial effects of mechanical stimulation on cartilage explant culture, 3) ZA can regulate the intracellular calcium signaling of chondrocytes, and 4) ZA regulates the mevalonate pathway of chondrocytes.

Methods: Rescue from Traumatic Damage: Cartilage allografts from 3-month old calf knee joints were cultured in serum supplemented medium (DMEM, 10% FBS, 1% P/S) for 1 week to simulate traumatic damage on cartilage caused by joint bleeding. Our previous studies showed that the mechanical integrity of cartilage explants was significantly impaired after long-term exposure to serum[3]. The samples were further divided into two groups and cultured in chemically defined serum-free medium[4] for 4 weeks with or without the supplement of 1 μM ZA. Longitudinal mechanical properties of the explants were measured, followed by GAG and collagen assay at the end of the 4-week culture. Expressions of aggrecan (AGN), type I collagen (COL1), type II collagen (COL2), ADAMTS5, and MMP-13 genes were measured using qRT-PCR after 2 weeks of culture.

Mechanical Stimulation: After harvesting, cartilage explants were cultured in serum-free medium with or without 1 μM ZA for 4 weeks. During the culture, samples were stimulated with mechanical loading (10% preload followed by ±3% dynamic loading) for 30 minutes every day. Mechanical properties, including equilibrium Young’s modulus and dynamic modulus, of explants were measured weekly. Gene expressions of aggrecan (AGN), type I collagen (COL1), type II collagen (COL2), ADAMTS5, and MMP-13 were tested after 2 weeks of culture. GAG and collagen contents were determined after 4 weeks. At day 2, 8, and 15, five extra cartilage explants were cut into two identical halves and stained with Fluo-8 AM. The spontaneous intracellular calcium signaling of in situ chondrocytes were recorded using a confocal microscope (Zeiss LSM510) and analyzed as previously described[5].

Related Signaling Pathways: After harvesting, the cartilage explants were separated into 2 groups: (1) chemically defined culture medium; (2) culture medium supplemented with 1 μM ZA. At days 2, 8, and 15, spontaneous intracellular calcium responses of in situ chondrocytes were recorded and analyzed. Another short-term cultured (3 days) batch of samples were separated into 3 groups to check the effects
of mevalonate derivatives: (1) chemically defined medium with 10 μM ZA and DMSO vehicle; (2) culture medium with 10 μM ZA and 5 μM farnesol (FOH); (3) culture medium with 10 μM ZA and 5 μM geranylgeraniol (GGOH). Both FOH and GGOH are derivatives of mevalonate, the presence of which may reverse the inhibition of mevalonate pathway. After 48 hours treatment, spontaneous intracellular calcium signaling of chondrocytes was recorded and analyzed.

**Results:** *Rescue from Traumatic Damage:*

For cartilage explants initially exposed to serum for 1 week, the Young’s modulus of the ZA rescued group increased more than 200% after 4 weeks of culture (Fig. 1A), while the non-ZA group showed a 56% increase (Fig. 1B). GAG content in the ZA group (Fig. 1C) was significantly higher than the non-ZA group, but no difference for the collagen content (Fig. 1D). Anabolic gene expressions of type I, II collagens were significantly higher in the ZA group than non-ZA group (Fig. 2F-H), and expressions of catabolic genes, ADAMTS5 and MMP-13 (Fig. 2I-J), were significantly lower in the ZA group.

**Mechanical Stimulation:**

Exposure to ZA benefited the cartilage explant culture with daily mechanical stimulation. ZA significantly increased the equilibrium Young’s modulus (Fig. 2A) and dynamic modulus (Fig. 2B) after 2 weeks, although no difference was detected in GAG (Fig. 2C) or collagen content (Fig 2D). The spontaneous calcium signaling of chondrocytes, represented by responsive percentage of cells, were higher in ZA treated group at D2 (p<.05), D8 (p<.01), and D15 (p<0.001) than those of non-ZA group. Expressions of type I, II collagen and aggrecan genes were significantly higher in the ZA group than the non-ZA group (Fig. 2F-H), and expressions of ADAMTS5 and MMP-13 genes (Fig. 2I-J) were significantly suppressed in the ZA group.

**Related Signaling Pathways:**

For the calcium signaling of in situ chondrocytes in cartilage during the long term culture, the responsive percentage of cells in the ZA group increased with culture time (Fig 3A), while the non-ZA group remained constant. Further analysis showed that the presence of ZA induced higher calcium peak magnitude, shorter time between neighboring peaks, and shorter calcium fluctuation time (data not shown). During the short-term treatment of ZA on cartilage explants, the mevalonate derivative FOH can reverse the effect of ZA. The calcium responsive percentage in the ZA+FOH group (25.0%) was lower (p<.001) than that of ZA vehicle group (33.7%), while no significant difference was detected between ZA+GGOH (33.2%) and ZA vehicle group.

**Discussion:** Several studies showed that bisphosphonate can suppress the development of PTOA in animal models, suggesting a promising therapeutic technique for trauma related cartilage degeneration. Our in vitro experiments proved that ZA can also rescue traumatic damage in cartilage explants, which implies that the chondro-protective effect of ZA is related to its direct action on chondrocytes. This conclusion is further strengthened by the fact that ZA treatment can promote the beneficial effects of mechanical stimulation during cartilage explant culture. Supplement of mevalonate derivatives FOH can reverse the effect of ZA on calcium signaling, but not GGOH, which suggests that the chondro-protective effect of ZA could result from the inhibition of the prenylation of small GTPases (Ras, Rho) in the mevalonate pathway; To be specific, impeding the protein farnesylation, which might inhibit the expression of chondrocyte degradation (collagenase) enzymes. ZA compromises the protein farnesylation, causing negative feedback upstream for HMG-CoA reductase, which is an integral protein of the endoplasmic reticulum (ER), a major storage of intracellular Ca^{2+}. It is conceivable that such
inhibition of HMG-CoA reductase could release Ca^{2+} from ER, activate phospholipase C (PLC), and ultimately lead to escalated intracellular calcium signaling activities.

**Significance:** This study identified a new targeting pathway for the prevention of cartilage degeneration after joint traumatic damage.
Figure 3: (A) Responsive percentage of the spontaneous $[\text{Ca}^{2+}]_i$ signaling of in-situ chondrocytes during 2 weeks culture; (B) Responsive percentage of the spontaneous $[\text{Ca}^{2+}]_i$ signaling after 48 hours treatment with ZA and the derivatives of mevalonate pathway. (*: $p<.05$, **: $p<.01$, ***: $p<.001$)