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The effect of human-like collagen calcium complex on osteoporosis mice

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ABSTRACT

The objective of this study was to assess the effect of a modified human-like collagen calcium complex on osteoporosis mice. BHK (*Baby Hamster Kidney*) cells were used to compare the cytotoxicity of different calcium reagents with the MTT test. Six-week-old male mice (n = 80) were randomly divided into eight groups: a blank group (blank), control group (control), human-like collagen calcium group (HLC-Ca), thiolated human-like collagen calcium group (SH-HLC-Ca), phosphorylated human-like collagen calcium group (Pi-HLC-Ca), gluconate group (Glc-Ca), calcium carbonate group ($CaCO_3$) and D-cal group (B). A systematic analysis of the results available *in vivo* after 3 months of treatment was presented. The effects of several Ca supplements on osteoporosis mice were investigated by detecting serum calcium, alkaline phosphate activity (ALP), bone hydroxyproline (BHP) and bone mineral density (BMD). The results proved that the BMD and BHP of osteoporosis mice were significantly increased in the Pi-HLC-Ca group, while serum calcium and ALP were decreased. Therefore, Pi-HLC-Ca is likely a good calcium supplement for clinical applications. In this review, the advantage of Pi-HLC-Ca in preventing and delaying osteoporosis is highlighted. In addition to the current progress, further investigations are necessary to reveal the relative influences of collagen and calcium proportions on the long-term clinical effects of osteoporosis.

1. Introduction

Calcium (Ca) plays a vital role in the life of human beings, as one of the most important minerals in the human body. However, calcium deficiency is also very common and can lead to osteoporosis and osteopenia, which are always related to calcium deficiency [1,2]. Calcium reagents can be used to restore the balance between resorption and formation [3]. In addition, calcium supplementation is regarded as a safe monotherapy method for osteoporosis [4]. However, even long-term supplementation with calcium only postpones bone loss and provides no favourable effect on the preservation of bone mineral density [5,6].

Moreover, collagen, the most abundant protein, plays an important role in the body. Collagen is not only an important part of bone tissue but also an essential part of bone health; for instance, type I collagen is a primary organic component that makes up the extracellular matrix of bone [7–9]. Collagen, a biocompatible material, is widely used in interventions due to its low risk of immunological reactions [10]. Human-like collagen (HLC), which is prepared in our laboratory to be adopted as a biological material, is also widely used, including as bone materials and hydrogels that show good compatibility and that stimulate no immunological effect [11–14]. It is also known that collagen or hydrolysates *in vivo* can generate peptides that are useful for organic biosynthesis [15,16]. It has been shown in some studies that oral administration of hydrolysed collagen may improve bone mass content and density in rats [17]. In general, unexpected results may be achieved through combining human-like collagen with calcium.

Bone, a dynamic organization, is constantly updating and remodelling to maintain its integrity [18–20]. In other words, newly generated bone is constantly replacing the old bone and is accommodating the mechanical load changes [21]. Once osteoclasts are attached to the bone surface, protons and proteolytic enzymes are secreted, which may dissolve the bone mineral and hydrolyse the matrix protein. Then, osteoblasts replace the osteoclasts to capture protons and protein to form calcium and phosphate deposits [22].

The specific process is shown in Fig. 1. In this process, the old bones

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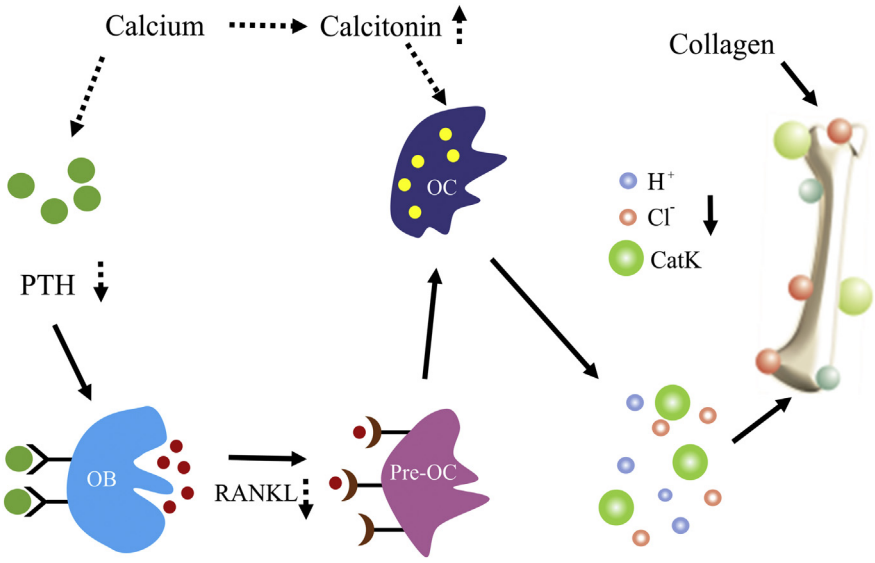

Fig. 1. Calcium supplementation could reduce parathyroid hormone (PTH) levels, resulting in diminished RANKL (receptor activator for nuclear factor-κB ligand) expression by osteoblasts. The content of cathepsin K (CatK) and protons that the osteoclasts captured on the bone surface were reduced. Thus, the matrix proteins (principally the type I collagen) and bone mineral were protected.

are updated and replaced to maintain skeletal balance. However, in elderly persons, the balance between formation and resorption shifts towards resorption. If the unbalanced state continues, in the long-term, calcium, bone mineral density and matrix protein will decrease, leading to osteoporosis. Osteoporosis, a common disease, is characterized by low bone mineral density and an increase in the risk of fractures of the hip, spine, and forearm [5,23]. Age is a neglected risk factor for osteoporosis. Osteoporosis may be prevented or delayed by maximizing peak bone mass during adolescence.

The aim of this study was to evaluate the effect of the human-like collagen calcium complex (prepared by our laboratory) on osteoporosis mice, which are produced by oral overdoses of retinoic acid [24,25]. The primary endpoints of the study were to alleviate mouse bone disorders, such as low BMD and high serum calcium. Future studies may suggest the importance of clinical examinations as well as a way to prevent or delay osteoporosis. People suffering from calcium deficiency and osteoporosis will be treated, or the condition will be prevented, to alleviate the burden on patients and society.

2. Materials and methods

2.1. Materials

Human-like collagen (HLC, China patent number: ZL01106757, M_r = 97,000) was supplied by Xi'an Giant Biogene Technology company. HLC-Ca, SH-HLC-Ca and Pi-HLC-Ca were prepared by our laboratory. Glucose calcium (Glc-Ca) was purchased from Sanchine. $CaCO_3$ and D-cal (B) were purchased from Anshi Pharmaceutical Inc. All the reactants and solvents used were of analytical grade, and double-distilled water was used throughout the experiments.

2.2. Preparation of HLC-Ca, Pi-HLC-Ca and SH-HLC-Ca

Preparation of HLC-Ca: Freeze-dried HLC was dissolved in ultrapure water at a concentration of 10 mg/mL, the pH was adjusted to 7.5, and $CaCl_2$ solution was added into the HLC solution (n (HLC): n ($CaCl_2$) = 1:1000), followed by gentle stirring at room temperature for 1–1.5 h. Finally, the reaction mixture was purified using dialysis and freeze-dried in a vacuum freeze drier (FD5-10, SIM, US).

Preparation of Pi-HLC-Ca: Then, the freeze-dried HLC was dissolved in ultrapure water at 10 mg/mL, and G-6-P-Na_2 (10 mg) was added to the HLC solution (pH 8.0–8.5), After incubating for 2–4 h in a 50 °C water bath, the Pi-HLC was prepared and then freeze-dried. The freeze-dried Pi-HLC was dissolved in a saturated KI solution at 55 °C; then, freeze-dried Pi-HLC was dissolved in MOPS buffer (10 μM, pH 7.5), and $CaCl_2$ solution was added into the Pi-HLC solution (n (Pi-HLC): n ($CaCl_2$) = 1:600), followed by gentle stirring at room temperature for 1–1.5 h. Finally, the reaction mixture was purified and freeze-dried.

Preparation of SH-HLC-Ca: SH-HLC was prepared by adding S-AMSA to HLC solutions. Then, freeze-dried SH-HLC was dissolved in ultrapure water at a concentration of 10 mg/mL, and $CaCl_2$ solution was added into SH-HLC solution (n (SH-HLC): n ($CaCl_2$) = 1:1000), followed by gentle stirring at room temperature for 1 h. Finally, the reaction mixture was purified and freeze-dried.

2.3. Cell culture and cell viability

BHK (*Baby Hamster Kidney*) cells (CBCAS, Shanghai, China) were cultured in RPMI 1640 medium. The cytotoxicities of HLC, HLC-Ca, SH-HLC-Ca and Pi-HLC-Ca were assessed using the BHK cells. The samples were dissolved in cell culture at 20 mM (Ca^{2+}) and sterilized with a 0.22 μm filter. The cells were cultured in a CO_2 (5%) incubator at 37 °C on 96-well plates with 200 μL per well (1.0×10^4 cells·mL^{-1}) for 24 h and then were cultured with the sample solution for 24 h, 36 h and 48 h.

At the end of the incubation, 20 μL MTT (0.5%) was added to each well for another 4 h. The medium was removed, and DMSO (100 μL) was added to each well. Finally, the absorbance was measured at 490 nm. The relative cell growth (%) was calculated as follows:

$$\text{Relative cell growth (\%)} = OD_{test}/OD_{control} \times 100\% \quad (1)$$

where OD_{test} means the absorbance value of the test sample and $OD_{control}$ means the absorbance value of the blank sample. All tests were repeated three times, and we reported the average value of five parallel samples.

2.4. Cell fluorescence staining test

A cell fluorescence staining technique was used to observe apoptosis in the cells. DAPI (2-(4-amidinophenyl)-6-indole carbinol dihydrochloride) is a kind of blue fluorescence dye that can penetrate the cell membrane and bind to DNA; then, the cells exhibit a green colour when excited in blue light.

BHK cells were seeded at a density of 1.0×10^3 cells/cm^2 on 48-well plates and were cultured in RPMI 1640 medium in a CO_2 (5%)