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焦磷酸鈣添加焦磷酸鈉於治療骨質疏鬆症之研究(1/2)

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焦磷酸鈣添加焦磷酸鈉於治療骨質疏鬆症之研究

A Study of Bioactive Ceramic $\text{Ca}_2\text{P}_2\text{O}_7$ with $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ Addition on Treatment of Osteoporosis

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主持人：台大醫學院骨科 孫瑞昇醫師

一、中文摘要

關鍵詞：骨質疏鬆、福善美、SDCP 焦磷酸鹽、血清、組織學觀察、MTT assay、TUNEL 染色法、SEM、TEM。

骨質疏鬆症的臨床症狀是身體骨組織質量的減少和顯微結構的破壞而導致骨折危險性的增加。停經後由於抑鈣素和動情激素分泌的減少是造成骨質疏鬆症的主要危險因子，導致骨質流失大於形成速率。治療停經後骨質疏鬆的新藥-福善美(FOSAMAX[®])氨基雙磷酸鹽類藥物，在近年來扮演的角色越顯重要。

於本計劃前一年先期研究中我們評估福善美與SDCP 焦磷酸鹽對骨質疏鬆的效果。研究結果由骨灰燼觀察發現，SDCP 比 FOSAMAXR 更有效地提高骨質密度(BMD)。使疏鬆骨之骨小樑數目提高，可大幅提高骨強度。進一步於本計劃第一年使用體外實驗，利用 MTT assay、TUNEL 染色法，電子顯微鏡(SEM、TEM)分別評估 SDCP 焦磷酸鹽對蝕骨細胞活性、形態與凋亡現象。

結果顯示於 10^{-4}M SDCP 濃度下，骨母細胞數目有明顯增加之現象、而蝕骨細胞數目則有明顯減少之現象。使用免疫螢光反應檢驗顯示蝕骨細胞之凋亡有明顯增加之現象，電子顯微鏡評估顯示高基式體呈顯破壞病分散至細胞質中、稍後蝕骨細胞之細胞核呈顯濃縮及週邊化現象，此外在細胞凋亡之晚期出現細胞核膜破壞且細胞核內容物露出現象。至此我們可認為 SDCP 導致蝕骨細胞之凋亡主要以細胞核顯微結構之破壞及細胞內容之崩解為主。

本計劃第二年將使用蝕骨細胞標的蛋白如 OPG、OPG-L、Calcitonin receptors (CTR) mRNAs 之 cDNA 探針，探討蝕骨細胞與各種不同 SDCP 焦磷酸鹽生醫材料之生物反應。

經由此實驗，各種不同 SDCP 磷酸鈣生醫材料對骨細胞之影響，SDCP 焦磷酸鹽與蝕骨細胞間之生化及分子關係可以得到釐清。而 SDCP 焦磷酸鹽是否具有骨質疏鬆症臨床使用的潛力也可以獲得確定。

二、英文摘要

Keywords : osteoporosis, FOSAMAX[®], SDCP, serum, histological observation, MTT assay, TUNEL stain, SEM, TEM

Osteoporosis is a disease with clinical symptoms

of bone mass loss and deterioration of microarchitecture on body skeleton, which results in an increased risk of fractures. The loss of calcitonin and estrogen production in menopause is the major risk factor for osteoporosis, causing increased skeletal resorption and relatively decreased bone formation. Bisphosphonates have recently gained an increasing role in the management of osteoporosis. The aminobisphosphonate, FOSAMAX[®] (alendronate sodium, MSD) has recently been introduced as a new agent for the treatment of postmenopausal osteoporosis. The purpose of this study is to evaluate the effect of FOSAMAX[®] and SDCP on osteoporosis.

In the pilot study, we have found that the bone ashes increased significantly after ingestion of SDCP which is even higher than that of the Fosamax ingestion group. The bone trabeculae and interconnection between trabeculae were also increased significantly which mean that the strength of bone architecture also increased significantly. In the following year, we will evaluate the in-vitro effect of SDCP on the osteoclasts bone cell activities. The cellular morphology, viability and apoptosis of the osteoclasts and osteoblasts will be evaluated by the methods of MTT assay, TUNEL stain, scanning electron microscope (SEM), and transmission electron microscope (TEM) analysis, respectively.

The results showed that at 10^{-4}M SDCP, the osteoblast cell count increased significantly, while the osteoclast population decreased significantly. Apoptosis of the osteoclast population was well demonstrated by immuno-histochemical study. Ultra-structural study showed that the Golgi apparatus was degraded, or dispersed in the cytoplasm. Later, osteoclasts revealed pyknotic nuclei showing condensation and margination of heterochromatins and DNA fragmentation, which were typical features of apoptosis. In addition, disruption of nuclear envelopes leading to leakage of nuclear contents into the cytoplasm was observed in the late stage of apoptosis. We concluded that SDCP induces apoptosis of osteoclasts was characterized by ultrastructural changes of the nucleus accompanied by degradation of cellular organelles.

In the second year's study, we will use these markers of the osteoclasts, including the mRNAs

of OPG, OPG-L, Calcitonin receptors to evaluate the molecular and biological response of the osteoclasts to the SDCP biomaterials.

Through this project, the molecular and biological response of bone cells to SDCP biomaterials will be established. The model of biological response of bone cells to SDCP biomaterials will be elucidated. The possible clinical application of SDCP in the osteoporosis will be established.

INTRODUCTION

Osteoporosis is a major health-care problem of ageing communities characterized by an increase in bone resorption relative to bone formation. The progressive decrease in bone mass leads to an increased susceptibility to fractures, which result in substantial morbidity and mortality. Although there are several risk factors for fractures, reduced bone mineral density is the strongest predictor. Thus, the ultimate goal of pharmacologic treatment in osteoporosis is to reduce the risk of fractures by increasing bone mass of normal quality.

Osteoclasts play a key role in bone resorption and bone remodeling in conjunction with osteoblasts. Recently, Hughes et al. have demonstrated that an injection of bisphosphonates efficiently caused apoptosis of osteoclasts (Hughes et al. 1995). Sintered dicalcium pyrophosphate is a synthetic compound that has been proved to be quite biocompatible to bone tissue in the in-vivo animal model (Lin et al. 1995). In the in-vitro model, Sintered dicalcium pyrophosphate has been proved to be more biocompatible than hydroxyapatite (Sun et al. 1997). Recent work in our institute has demonstrated that the ingestion of either bisphosphonate or sintered dicalcium pyrophosphate decreased the bony porosity and increased bone mineral contents in the long bones of ovariectomized rats (Sun et al. 2002). In this study, we use an in-vitro cell culture model to investigate ultrastructural changes and fate of osteoclasts undergoing apoptosis induced by sintered dicalcium pyrophosphate.

MATERIALS AND METHODS

Preparation of sintered β -dicalcium pyrophosphate solutions

The powder of sintered β -dicalcium pyrophosphate (SDCP) was prepared as previously described (Lin et al. 1995). The obtained sintered dicalcium pyrophosphate was dissolved by HCl and then diluted into different concentration of solutions by culture medium used in the bone cell culture. In the first part of this study, the effects of various concentration of sintered dicalcium pyrophosphate on bone cell activities were evaluated by using MTT assay as described below. Four different concentrations (1.0×10^{-2} M, 1.0×10^{-3} M, 1.0×10^{-4} M, 1.0×10^{-5} M) were tested for 1 day, 3 days and 7 days period.

Osteoblast/Osteoclast Bone Cell Culture

The rat alveolar mononuclear cells-calvarias osteoblasts co-culture system used in this study was the same as previously described (Sun et al. 2000). The culture dishes were incubated at 37°C in an atmosphere supplemented with 5% CO_2 . After one week's culture, the cells grew to 80% confluence, various concentrations of sintered dicalcium pyrophosphate were added.

Colorimetric MTT (Tetrazolium) assay for cell viability

The mitochondria activity of the bone cells after exposure to various concentrations of SDCP was determined by colorimetric assay which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma catalog no. M2128, Sigma Co., St. Louis, MO, USA) to formazan.

Identification of Fragmented DNA by TUNEL Staining

Detection of apoptosis in bone cells after SDCP treatment were performed by the immunohistochemical methods. The DNA strand breaks in bone cells were then fluorescence-labeled by 100 μl TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture (Boehringer Mannheim, Mannheim, Germany). amples were then analyzed under light microscope.

Ultrastructural Study

For transmission electron microscopy (TEM), the specimens were stained with uranylacetate and lead citrate were examined under a Zeiss EM EM109 electron microscope (Zeiss Co. Ltd., Germany) operated at 80 kV.

RESULTS

Quantitative Analysis of Osteoblasts and Osteoclasts

When osteoblast cells cultured with 10^{-2}M or 10^{-3}M concentrations of SDCP, the osteoblasts cell count was significantly decreased; while in the 10^{-4}M or 10^{-5}M concentration of SDCP, the cell count of osteoblasts increased significantly and this effect persisted till the end of 3 days' culture. At the 10^{-4}M concentration of SDCP, the beneficial effect on the osteoblasts persisted till the end of 7 days' culture, although it did not attain the significant level.

When osteoclast cells cultured with 10^{-2}M , 10^{-3}M or 10^{-4}M concentrations of SDCP, there was significant decrease in the cell count of osteoclasts; while at the 10^{-5}M concentrations of SDCP, there was no statistically change in the cell count of osteoclasts.

Immuno-Histochemical Identification of Apoptosis

After 1 day's SDCP treatment on bone cells, the apoptosis of the osteoclasts was detected in the osteoclasts clusters by showing dense stain in the samples. The apoptosis of osteoclasts were even more significant after 3 days' treatment of

SDCP as manifested by the pyknotic changes in the nucleus of the osteoclasts giant cells. After 7 days' SDCP treatment, there is loss of cellularity in the center of osteoclasts giant cells and some residual apoptotic cells with pyknotic nucleus visible, but the surrounding osteoblasts populations were relatively preserved.

Ultrastructural Changes by Electron Microscopic Examinations

Osteoclasts in the day 1 of apoptosis, the Golgi apparatus was degraded, resulting in accumulation of small vesicles in the area of Golgi apparatus, or dispersed in the cytoplasm. At 3 days, osteoclasts later exhibited typical features of apoptosis with numerous pyknotic nuclei containing condensed heterochromatins, which were often marginated against the nuclear envelope. The Golgi apparatus was not observed, and there were many vesicles in the cytoplasm. These osteoclasts were often convoluted to give rise to membrane-bound apoptotic bodies on day 3. The pyknotic nuclei of osteoclasts in the day 7 of apoptosis displayed enlargement of the nuclear envelope resulting from dissociation of the outer and inner nuclear membranes with accumulation of amorphous material between them.

In most of severely apoptotic osteoclasts at day 7, some nuclei were disrupted, as evidenced by the rupture of nuclear envelope, leading to leakage of nuclear components in the cytoplasm.

DISCUSSION

Osteoporosis is a major health-care problem of ageing communities. For the evaluation of agents intended to prevent and/or treat postmenopausal osteoporosis, the adult ovariectomized rat is a convenient and reliable experimental model. In the pilot study of our institute, we have proved that the sintered dicalcium pyrophosphate can increase bone mass in the ovariectomized rat (**Sun et al. 2002**). It is difficult to exam in-vivo reaction of a specific cell to the substrate because numerous cell populations and chemical factors are involved. In order to determine the sequences of events and the parameters influencing the interactive process, the model of cell culture in the presence of specific substrate is of great importance. In this study, we used an in-vitro bone cell culture model to investigate the ultra-structural changes and fate of osteoclasts after sintered dicalcium pyrophosphate administration.

From this study, we can summarized that sintered dicalcium pyrophosphate, unlike bisphosphonate, has high affinity to osteoblasts. Sintered dicalcium pyrophosphate induced osteoclasts apoptosis that was characterized by degradation of the Golgi apparatus followed by nuclear pyknosis associated with condensation and margination of chromatin and formation of apoptotic bodies. Enlargement and fusion of the nuclear envelope were characteristic of apoptosis of osteoclasts. Because apoptotic osteoclasts

induced by sintered dicalcium pyrophosphate resemble dying or dead osteoclasts seen in a physiological condition, osteoclasts may be subjected to cell death by apoptosis.

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