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# Inhibitory effects of beta-tricalciumphosphate wear particles on osteocytes via apoptotic response and Akt inactivation

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ABSTRACT

Wear debris-induced osteolysis, a major contributing factor of orthopedic implant aseptic loosening, affects long-term survival of orthopedic prostheses following joint replacement and revision surgery. Pathogenic effects of wear debris on various cell types including macrophages/monocytes, osteoblasts, and osteoclasts have been well studied. However, the interactions between wear debris particles and osteocytes, which make up over 90% of all bone cells, have not been clearly illustrated. Here, we explored the biological effects of endotoxin-free beta-tricalciumphosphate (β-TCP) wear particles with the average diameter of 1.997 μm (range 1.3–3.2 μm) on osteocytes in vitro. Our results showed that 24 h or 48 h incubation of β-TCP particles dose-dependently inhibited cell viability of osteocytes MLO-Y4. Alternatively, β-TCP particles treatment for 24 h significantly increased the osteocytic marker SOST/sclerostin mRNA expression and the release of inflammatory cytokines including TNF-α and IL-1β into the culture media, but decreased the mRNA expression of another osteocytic marker dentin matrix protein-1 (DMP-1). Furthermore, these osteocytes dysfunctions were accompanied by F-actin disassembly, cell apoptosis, sustained enhancement of intracellular reactive oxygen species (ROS) and mitochondrial injury upon β-TCP particles stimulation. In addition, β-TCP particles also caused Akt inactivation at Ser473 resides with a dose- and time-dependent pattern. Taken together, β-TCP wear particles could cause osteocytes dysfunctions, which may be mediated by apoptotic death and Akt inactivation in MLO-Y4 cells. These findings strongly suggest that osteocytes may play an important role in the β-TCP wear particles-induced osteolysis, and provide valuable insights for understanding the molecular mechanisms of osteocytes death involved in tissue damage during bone cement and intolerance of cemented prostheses.



## 1. Introduction

Joint arthroplasty is one of the most successful bone repairs for joint diseases, as it often relieves pain excellently and improves quality of life for individuals with moderate to severe osteoarthritis. However, some implants are less satisfactory because of loosening, and require revision arthroplasty. A recent study projected the growth of total hip and total knee revisions is 137% and 601%, respectively, between 2005 and 2030 (Kurtz et al., 2007). Late aseptic loosening is the most predominant cause for implants failure (Bordenave et al., 2005), which causes significant morbidity and accounts for substantial health care expenditure.

Late aseptic loosening, as one of the major complications for joint arthroplasty, is mostly produced by wear particles generated from prosthetic implants materials such as polymers, metal and bone cements. Wear particles can activate a wide variety of cell types within the periprosthetic soft tissue including fibroblasts, macrophages, osteoblasts, and osteoclasts, which release TNF-α, IL-6 or IL-1β, and other pro-inflammatory cytokines (AlSaffar and Revell, 1994; Schwarz et al., 2000; Kaufman et al., 2008; Shimizu et al., 2010). These pro-inflammatory mediators in turn directly or indirectly stimulate osteoclasts attachment, differentiation, activation and maturation, leading to bone resorption within peri-implants and possible implant failure (Grandjean-Laquerriere et al., 2005; Lange et al., 2009).

In addition to inflammatory cytokines responsible for wear particles-induced osteolysis, some recent in vivo and in vitro studies showed that direct or indirect induction of cell death was involved in the wear particles-induced bone resorption (Ciapetti et al., 2002; Yang et al., 2002; Haleem-Smith et al., 2011). For example, ultra-high molecular weight polyethylene (UHMWPE) particles were implanted in a vivo murine model of inflammation for 7 days, and triggered apoptotic responses. The number of apoptotic cells is associated with particles shapes, as the more severe apoptotic changes provoked with the elongated particles than those of the

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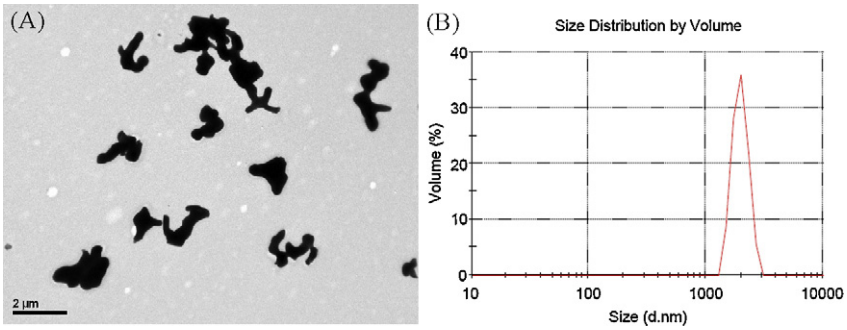


**Fig. 1.** SEM image of β-TCP wear particles (A) and DLS assay for their distribution size (B). Bar = 2 μm.

globular particles (Yang et al., 2002). Other studies also demonstrated that the potential for orthopedic wear particles of metal and bone cements to cause apoptosis of several types of cells including human mesenchymal stem cells, osteoblast-like cells of MG-63 and HL-60 cells (Ciapetti et al., 2002; Haleem-Smith et al., 2011).

The apoptotic process consists of sequential cellular events. Except for the degradation of nuclear DNA and F-actin disassembly, mitochondrial injury has confirmed to be involved in apoptosis (Kachadourian et al., 2010). Some apoptotic stimuli opens mitochondrial permeability transition pore (mPTP), which can induce loss of mitochondrial membrane potential ($\Delta\psi_m$), outer membrane rupture, ROS release and other apoptosis initiation factors into the cytoplasm. Intracellular excess of ROS accumulation in turn directly causes the opening of mPTP and starts a series of apoptotic responses. Therefore, mitochondrial injury may be an early event in regulation of apoptosis. However, the role of ROS and mitochondria in the wear debris-induced apoptosis remains unknown.

Furthermore, recent reports of Smith and Beidelschies (Smith et al., 2007; Beidelschies et al., 2008) both indicated that Ti particles induced Akt activation in cultured RAW264.7 murine macrophages, which was accompanied by an obvious increase of TNF-α production. These observations suggest that Akt signaling pathways may be responsible for potentiation cytokine production, osteoclast differentiation, and bone resorption induced by wear particles.

Osteocytes, making up over 90% of all bone cells, are embedded in lacunae within the mineralized matrix. They are derived from osteoblasts on the bone surface, and form a syncitial network to communicate with neighboring osteocytes and other cells at the bone surface via cell processes within canaliculi to convey local signals directly to osteoblasts or osteoclasts (Nakashima et al., 2011). As for the role of osteocytes in the wear debris-induced osteolysis, Lohmann et al. (2002) reported that 24 h treatment of UHMWPE caused 30% cell death, and significantly increased the level of prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO) in the culture media of osteocytes MLO-Y4. Co–Cr–Mo alloy particles also significantly triggered osteocytes apoptotic death, which was partially (40%) dependent on TNF-α production (Kanaji et al., 2009). Then, osteocytes may be involved in aseptic loosening by affecting macrophages and osteoblasts as well as osteoclasts that cause bone resorption (Ciapetti et al., 2002; Haleem-Smith et al., 2011; Wing-Yee et al., 2012). But there is no investigation of the interactions between β-TCP wear particles and osteocytes, and its possible molecular mechanism.

In this study, we chose commercial β-TCP particles to simulate β-TCP wear debris from loosened β-TCP implants, which have been widely used as bone substitute biomaterials in orthopedic and dental surgery for almost 40 years, and investigated the direct biological effects of the particles on osteocytes, mainly focusing on osteocytes dysfunctions, mitochondrial changes, apoptosis and Akt signaling.

## 2. Materials and methods

### 2.1. Chemical and materials

Alpha-Minimum Essential Medium (α-MEM), acetoxymethyl ester of calcein (Calcein-AM), Annexin V/PI apoptosis detection kit, MitoTracker Red FM, JC-1, MTT and TRIzol® SuperScript™ First-Strand Synthesis System for RT-PCR were from Invitrogen Corp. (Carlsbad, USA). Fetal bovine serum (FBS) and calf serum (CS) were obtained from Genetimes Technology, Inc. (Shanghai, China). The mouse antibodies including anti-Akt1/2 nd anti-p-AktSer473 were purchased from Cell Signaling Inc. (Cell Signaling Technology, USA). Mouse antibodies for anti-caspase 3 and anti-β-actin were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Rat tail collagen I, TUNEL kit, 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) and FITC-labeled phalloidin were from Beyotime Institute of Biotechnology (Shanghai, China). TMB Liquid Substrate kit was from Amresco, Inc. (Solon, USA). The SYBR® Green stain was from BioWittaker Molecular Applications (Rockland, USA). The ELISA detection kits of IL-1β and TNF-α, E-Toxate-Kit, the secondary antibody and other biological reagents were from Sigma–Aldrich Inc. (St. Louis, USA). Other chemicals were AR expect for special instructions.

### 2.2. The particles characterization

Commercial pure β-TCP microparticles were used in this study (Ensail Beijing Corp., Beijing, China). Scanning electron microscopy (SEM Sirion 200, FEI Company, Dutch) image and Dynamic Lighting Scattering (DLS) assay (Zetasizer2000, Malvern, England) indicated that the average size of β-TCP particles was 1.997 μm (range 1.3–3.2 μm, Fig. 1) with forming aggregates of various sizes, and the particles have negative charge (−11 mV). The particles, autoclave at 120 °C for 20 min, were tested with the E-Toxate-Kit (Sigma, St. Louis, USA), indicating endotoxin levels below the detection limit of 0.05 U/mL. We therefore could exclude contamination of β-TCP particles with endotoxins. Prior to use particles were incubated in fresh 70% ethanol with shaking overnight. The particles were rinsed and suspended in endotoxin-free phosphate buffered saline (PBS) to a stock concentration of 5 mg/mL.

### 2.3. Culture of osteocytes MLO-Y4

MLO-Y4 osteocyte-like cells were kindly provided by Dr. Lynda Bonewald (University of Missouri-Kansas City, MO, USA). The MLO-Y4 cells were cultured on rat tail collagen I coated surfaces with α-MEM media supplemented with 2.5% FBS, 2.5% CS and antibiotics. Cells were plated at a density of $1.0 \times 10^5$ cells/well in the culture plates. After 24 h incubation, the MLO-Y4 cells were treated with β-TCP particles (0, 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL) for the desired time without subsequent media change.

### 2.4. Cell viability assay

The cell viability was observed by the fluorescence of Calcein-AM staining in viable cell. In brief, osteocytes MLO-Y4 with or without β-TCP particles treatment for 48 h were washed twice in PBS and incubated with 5 μM Calcein-AM for 15 min at 37 °C. The grafts were washed and visualized with a LSM 510 confocal laser microscope (Carl Zeiss, Germany) at excitation/emission wavelength of 488/517 nm.

In addition, MTT was used to quantitatively measure the effect of β-TCP particles on the viability of osteocytes MLO-Y4. The assay exploited the ability of mitochondrial dehydrogenases in metabolically active cells to reduce the yellow MTT, generating a purple formazan product. The MLO-Y4 cells were cultured in the presence of β-TCP particles (0, 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL) in standard 96-well plates for 24 h and 48 h. MTT reagent (5 mg/mL) was added to each cell