Biological response of Co^{2+} to preosteoblasts during aseptic loosening of the prosthesis

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Abstract

BACKGROUND: Aseptic loosening of prosthesis is the main long-term complication after artificial joint replacement. Metal ions have been proven to be one of the causes of aseptic loosening. How to control and mitigate aseptic loosening is an issue of concern.

OBJECTIVE: To observe the biological response of preosteoblasts challenged with Co^{2+} during aseptic loosening of the prosthesis.

METHODS: (1) *In vitro*: Preosteoblasts (MC3T3-E1) of mice were co-cultured with osteoblast induction solution of mice containing different concentrations of Co^{2+} for 72 hours, respectively, and induced into osteoblast cells. The cell proliferation was tested by MTT assay and the cytotoxicity of different concentrations of Co^{2+} was measured with the activity of lactate dehydrogenase. The concentration of alkaline phosphatase protein was used to detect the transformation ability of preosteoblasts. RT-PCR was performed to detect the mRNA expression of related factors. (2) *In vivo*: titanium nails were implanted into the proximal tibia of mice. The mice were divided into three groups. Mice in the stable control group were implanted with titanium nails. Mice in the loosening control group were implanted with titanium nails and cobalt-chromium particles. Mice in the cobalt ion group were implanted with titanium nails and cobalt-chromium particles and injected with cobalt-stimulated preosteoblasts. Bone mineral density around prosthesis was detected by MicroCT scanning immediately after surgery. Five weeks later, the bone density around the prosthesis was measured again. The mice were sacrificed and the affected knee joints were dissected for the pull-out test. The tissue after nail pull was stained with hematoxylin and eosin. The looseness of the prosthesis was determined by the force of the nail pull. The degree of inflammation was reflected by the thickness of the membrane between the prosthesis and the bone interface. The number of osteoclasts in the tissues around the prosthesis was observed by anti-tartrate acid phosphatase staining.

RESULTS AND CONCLUSION: (1) *In vitro* results: As the concentration of Co^{2+} increasing, the proliferation of preosteoblasts was decreasing. Co^{2+} had a significant inhibitory effect on serum alkaline phosphatase expression by preosteoblasts. Co^{2+} promoted monocyte chemoattractant protein-1, tumor necrosis factor-α, interleukin-6, receptor activator of nuclear factor κB ligand, nuclear factor of activated T cells c1 mRNA expression, and inhibited osteoprotegerin and osteoblast specific transcription factor Osterix mRNA expression. Low concentrations of Co^{2+} (62 μmol/L) promoted low density lipoprotein receptor-related protein-5 and Runx2 mRNA expression, but high concentrations of Co^{2+} (500 μmol/L) inhibited their expression. (2) *In vivo* results: MicroCT scan showed that the mice in cobalt ion group had the lowest bone mineral density ($P < 0.05$). In the cobalt ion group, the shear force required for pull-out test was significantly lower than that in the control group ($P < 0.05$). Hematoxylin-eosin staining showed that the formation of periprosthetic inflammatory reaction membrane was significant in the cobalt ion group; stimulation of preosteogenic cells by bivalent cobalt ions may exacerbate the inflammatory response around the prosthesis. (3) These results indicated that osteoblasts can play an important role in the aseptic loosening of the prostheses. Co^{2+} stimulated preosteoblastic cells play an important regulatory role in the differentiation and maturation of osteoclasts.

Key words: preosteoblast; metal ions; wear particles; aseptic loosening; inflammatory factors; osteoclasts; tumor necrosis factor; interleukin-6; alkaline phosphatase; tissue engineering

INTRODUCTION

With the progress of medical technology, artificial joint replacement has become one of the important technical means in the treatment of osteoarthrosis[1-2]. Aseptic loosening of the prosthesis is also the most common complication despite continuous improvements in the design concept and materials of prosthetic joint prostheses[3]. Numerous studies have shown that periprosthetic osteolysis induced by wear particles is the main cause for aseptic loosening[4-6]. Common wear particles include titanium alloy (Ti-6Al-4V), polymethyl methacrylate, ultra-high molecular weight polyethylene, and cobalt-chromium alloy (Co-Cr). LOCHNER et al.[7] found that wear particles of different materials can lead to different cellular biological responses and can activate a range of cytokines to affect the degree of osteolysis. Co-Cr alloy was widely used in prosthesis manufacturing because of its wear resistance, corrosion resistance and high temperature resistance, which ionic form included Co^{2+}

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and Cr^{3+}. Wear particles play a crucial role during periprosthetic osteolysis which are phagocytized by periprosthetic bone marrow mesenchymal stem cells, macrophages, osteoblasts, lymphocytes and fibroblasts, releasing cytokines and inflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α and PGE2 to act on osteoblasts and osteoclasts, respectively[8-10]. Wear particles can promote the activation and differentiation of osteoclasts, thereby promoting bone resorption, resulting in loss of bone mass and thus causing aseptic loosening of artificial prosthesis; it can inhibit the transformation of preosteoblasts into osteoblasts and formation of bone[11-12]. Whether metal ions can also cause joint prosthesis loosening is now a focus of concern, studies have shown that elevated levels of metal ions Co^{2+} and Cr^{3+} can often be detected in serum, urine and synovium of patients with long-term follow-up prosthesis replacement[13-14], and high concentrations of metal ions can lead to adverse biological reactions, including localized soft tissue toxicity, allergies, immune responses, inflammation, osteoporosis, and the risk of cancer[15]. Bivalent cobalt ions and trivalent chromium ions can increase the number of $CD8^+$ $HLA\text{-}DR^+$ T-cells and reduce the number of B-lymphocytes. Meanwhile, they can promote the secretion of TNF-α and IL-1β by monocytes and promote the production of osteoclasts[16-17]. However, most of these investigations have been focused on the relationships between metal ions and mature osteocytes; few studies have been done on the preosteoblasts. In this study, we first investigated the effects of Co^{2+} at different concentrations on the growth, differentiation, maturation, inflammatory response and osteoclast regulation of preosteoblasts *in vitro*. Finally, Pin-model was constructed to further verify the regulatory effect of Co^{2+} *in vivo*.

MATERIALS AND METHODS

Design

Cytological experiment and randomized controlled animal experiment.

Time and settings

This study was completed at Clinical Laboratory, Binzhou Medical College in May, 2018.

Materials

MC3T3-E1 cells were purchased from ATCC (CRL-2594), and divalent cobalt ion (Co^{2+}) was purchased from Sigma-Aldrich (Cat.#C8861). Eighteen female severe combined immunodeficient mice, aged 6 to 8 weeks, were provided by Shandong Experimental Animal Center, license No. SCXK (Lu) 20140007. Animal experiment was approved by the Ethics Committee of the Binzhou Medical University Hospital, approval number: 2018-003-01. Related information of cobalt ion is shown in **Table 1**.

Table 1 | **Information of cobalt ion**

Items	Information
Source	Sigma-Aldrich, St. Louis, MO, USA
Composition	Cobalt(II) chloride hexahydrate
Material appearance	Powder
Preparation	Dissolved in culture medium and sterilized with a 0.2 mol/L diameter filter
Physical and chemical properties	Red monoclinic system crystal, melting point 86 °C, soluble in water, and ethanol
Biocompatibility with the human body	Good

Methods

Culture and induction of preosteoblasts (in vitro)

MC3T3-E1 cells (ATCC, Manassas, VA, USA) were cultured in Alpha Minimum Essential Medium at 37% and 5% CO_2 atmosphere, which observed under a microscope, then the cells were fused and fell off to form cell suspension. After centrifugation and trypsin removal, the cells were subcultured in a ratio of 1 ∶ 3 and the third generation cells were taken for experiment. The MC3T3-E1 cells were cultured in Alpha Minimum Essential Medium containing 5% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin (Invitrogen), 100 mg/mL streptomycin (Invitrogen), 10 mmol/L β-glycerolphosphoric (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/mL L-ascorbicacid (Sigma-Aldrich), 100 nmol/L dexamethasone (Sigma-Aldrich) and different concentrations of Co^{2+} (Sigma-Aldrich) for 3 days[18-19].

Determination of MTT and lactate dehydrogenase (LDH) (in vitro)

MC3T3-E1 cells were seeded in 96-well culture plates and per well contains 8×10^3 cells. Osteoblasts inducing fluid (Sigma-Aldrich) mixed with 0, 62, 125, 250, 500, and 1 000 μmol/L Co^{2+} were added respectively. After 72 hours of differentiation induction, 20 μL MTT solution was added to each well to culture for 4 hours. After the supernatant was removed, 150 μL dimethyl sulfoxide (Invitrogen) was added and the absorbance of each well at OD_{450nm} was measured using a microplate reader. Cyto Tox 96®Non-Ratio Cytotoxicity Assay (Promega, Madison, WI, USA) was used to calculate the proportion of dead cells and viable cells. LDH assay was used to detect the toxicity of Co^{2+} at different concentrations to MC3T3-E1 cells.

Alkaline phosphatase (ALP) activity determination (in vitro)

The culture medium containing 200 μL preosteoblasts (MC3T3-E1) was inoculated into 48-well culture plate. After adding the osteogenic induction solution, the culture medium was co-cultured with two concentrations of Co^{2+} (62 μmol/L and 250 μmol/L) for 72 hours. The concentration of ALP protein in solution was determined by ALP Kit (Sigma-Aldrich) and the OD value was measured at 405 nm by spectrophotometer to reflect the content of ALP protein.

Real-time polymerase chain reaction (PCR) (in vitro)

RT-PCR was used to reveal the effects of different concentrations of Co^{2+} (62, 250 μmol/L) on the expression of monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-6, receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG), nuclear factor of activated T cells c1 (NFATc1), Runx2, osteoblast specific transcription factor Osterix (Osx) and low density lipoprotein receptor-related protein-5 (Lrp5) in MC3T3-E1 cells. Firstly, RNA was extracted from MC3T3-E1 cells which reacted with two different concentrations of Co^{2+} to determine the amount and concentration of RNA. After preparing the reverse transcription mixture, the reaction mixture was placed in Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) to prepare the cDNA. The detected genes were amplified by StepOnePlus® RT-PCR system (Applied Biosystems) and standardized with their respective housekeeping gene 18S.

Establishment of a model of joint loosening in mice (in vivo)

Eighteen female severe combined immunodeficient mice, aged 6–8 weeks, were fed adaptively for one week. The mice were isolated for two weeks before the experiment. Subsequently, a model of aseptic

loosening of mouse knee prosthesis was established (Pin-model) and the specific preparation method could be referred to the previous literature[20-21]. The standard model was that the titanium nail was completely implanted into the tibial medullary cavity of mice and the postoperative activity of the knee was not affected, which could be determined by postoperative X-ray examination and physiological activity of mice.

The Pin-model mice were randomly divided into three groups: (1) stable control group: titanium nail implantation (*n*=6); (2) loosening control group: titanium nail implantation+intra-articular injection of cobalt-chromium particles+MC3T3-E1 cells not stimulated by Co^{2+} (*n*=6); (3) cobalt ion group: titanium nail implantation+intra-articular injection of cobalt-chromium particles+MC3T3-E1 cells stimulated by Co^{2+} (*n*=6). MC3T3-E1 cells were induced by placing them in normal osteogenic induction fluid and osteogenic induction fluid containing 500 μmol/L Co^{2+}, respectively. One week after operation, 10 μL suspension containing two kinds of MC3T3-E1 cells was injected into the joint cavity of the loosening control group and the cobalt ion group (**Figure 1**).

MicroCT bone scan (in vivo)

The proximal tibia of 18 mice were performing microCT (vivaCT 40, SCANCO Medical, Brüttisellen, Switzerland) scanning to ensure that the titanium nails were located in the bone marrow cavity of the tibia after the operation, and the location of the titanium nail and the bone mineral density value of the proximal tibia were recorded postoperatively and 5 weeks after surgery, respectively.

Pull-out test (in vivo)

Mice were executed and the knee joints were amputated at the prosthesis joint to remove the tibia with titanium nails five weeks after operation. The cartilage around the tibial plateau was scraped with the surgical blade to expose the tail cap of the titanium nail. Bose 3200 ElectroForce load frame (Bose Corporation, Eden Prairie, MN, USA) was used for nail pulling experiment. The output data are analyzed and processed by Bose WinTest® (Bose Corporation) software.

Histological morphological evaluation and image analysis (in vivo)

After the nail pulling experiment, tibial tissue around the titanium nail was collected and decalcified to make paraffin sections. Hematoxylin-eosin staining was used to observe the degree of inflammation, bone formation and bone resorption in the tissue around the titanium nail under optical microscope. The number of osteoclasts was observed by TRAP staining. Six regions were randomly selected in each section to determine the number of osteoclasts with positive staining, and the positive cells of TRAP staining were counted according to Image-pro software (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemical assessments (in vivo)

Immunohistochemical staining was performed to detect proinflammatory cytokines (TNF-α) within the implanted tissue. The mice were sacrificed 5 weeks after surgery and the mouse limb bone chips were harvested and fixed in 10% formalin as soon as taken out from mice. The specimens were decalcified in 12% EDTA for 10 days followed by histology process to prepare 6 μm unstained sections. Immunohistochemical staining kits, polyclonal antibodies against human and mouse TNF-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Digital images were taken under a microscope and then analyzed using image-pro Plus software (version 7.0, Media Cybernetics, Silver Spring, MD, USA). Each picture was randomly selected from six regions to be counted.

Statistical analysis

Data are expressed as the mean ± SEM. For statistical analysis, rank sum test was performed using the GraphPad Prism 5.0 software package. Significance was set at $P < 0.05$.

RESULTS

Cytotoxicity of divalent cobalt ions and their effects on cell proliferation (*in vitro*)

Cells were cultured with Co^{2+} of different concentrations for 72 hours. MTT cell proliferation assay showed that the experimental groups which included 125, 250, 500 μmol/L Co^{2+} inhibited the proliferation of MC3T3-E1 cells in varying degrees ($P < 0.05$; **Figure 2**). LDH assay showed significant toxicity when Co^{2+} reached 1 000 μmol/L and Co^{2+} showed slight toxicity at 500 μmol/L ($P < 0.05$; **Figure 3**).

ALP activity (*in vitro*)

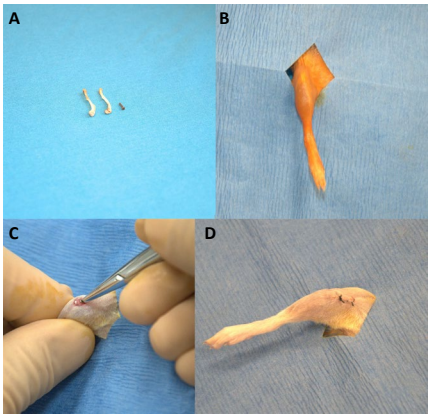
Figure 4 showed the different effects of two concentrations of Co^{2+} (62 μmol/L and 250 μmol/L) on ALP expression in MC3T3-E1 cells, respectively. When Co^{2+} was 250 μmol/L, it showed significant inhibition of ALP expression compared with the control group ($P < 0.05$).

Gene expression in preosteoblasts (*in vitro*)

RT-PCR was used to detect the expression of a variety of genes to further investigate the molecular biological mechanisms underlying the effects of different concentrations of Co^{2+} (62 μmol/L and 500 μmol/L) on MC3T3-E1 cells. The data showed that Co^{2+} significantly promoted MCP-1 gene expression at 500 μmol/L compared with the control group ($P < 0.05$). Both groups significantly promoted the expression of TNF-α and IL-6, and they also promoted the expression of RANKL. When Co^{2+} was at 500 μmol/L, the expression of RANKL was significantly increased compared with that of 62 μmol/L group. The expression of NFATc1 stimulated by Co^{2+} was significantly enhanced at 500 μmol/L compared with the control group ($P < 0.05$). The expression levels of OPG and OSX were inhibited in both experimental groups. When Co^{2+} was at 500 μmol/L, there were significantly differences with the control group; thus, the RANKL/OPG ratio was much higher than the control value ($P < 0.05$). Same trend with Lrp-5 and Runx2, both of them promoted the expression of Co^{2+} at 62 μmol/L and inhibited the expression at 500 μmol/L compared with the control group ($P < 0.05$; **Figure 5**).

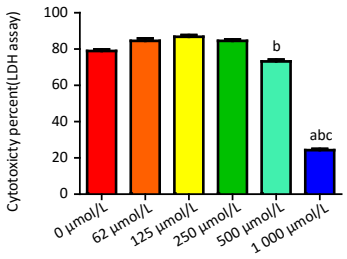
General situation and surgical results of experimental mice (*in vivo*)

The mice in each group grew and moved well, and generally responded well. No mice died in each group. The uneven distribution of cobalt and chromium particles around the prosthesis and the knee joint was observed after the knee joint was severed, and no significant anatomical changes were observed in the gross morphology of the knee joint (**Figure 6**).



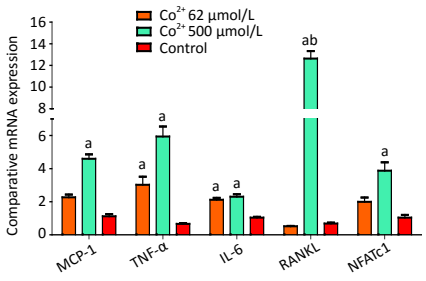
Note: (A) Pre-test materials. (B) External view of lower limbs after skin preparation in mice. (C) Titanium nails were placed in the proximal tibia of the mice. (D) External view of sutured soft tissue

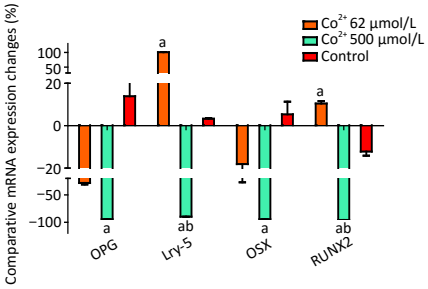
Figure 1 | Establishment of the model of knee prosthesis loosening in mice



Note: Cell viability and cytotoxicity were calculated by the relative medium LDH level over the cell lysis LDH (%). The left side of each group was the experimental control group. $^{a}P < 0.05$, *vs.* control group; $^{b}P < 0.05$, *vs.* 62 μmol/L group; $^{c}P < 0.05$, *vs.* 125 μmol/L group

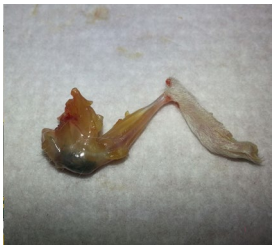
Figure 3 | Lactate dehydrogenase (LDH) assay to estimate the toxicity of MC3T3-E1 cells treated by Co^{2+} via serial concentrations





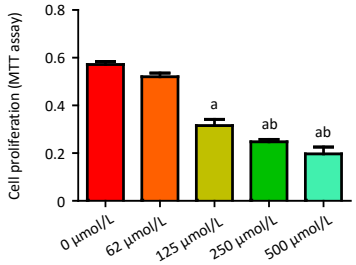
Note: Data show the expression of different genes compared with control group or different gene expression between the varying doses of Co^{2+}. The data obtained in Panel A were compared with the control group without Co^{2+} and expressed in relative gene copy numbers. The change in gene expression was represented by the percentage of Panel B relative to the control group, in which the control group was represented by baseline 0, the promoted expression was above the baseline, and the inhibited expression was below the baseline. MCP-1: Monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; RANKL: receptor activator of nuclear factor κB ligand; NFATc1: nuclear factor of activated T cells c1; OPG: osteoprotegerin; Lry-5: low density lipoprotein receptor-related protein-5; OSX: Osterix.$^{a}P < 0.05$, *vs.* control group; $^{b}P < 0.05$, *vs.* 62 μmol/L group

Figure 5 | RT-PCR to detect the gene expressing profiles in MC3T3-E1 cells after Co^{2+} induction



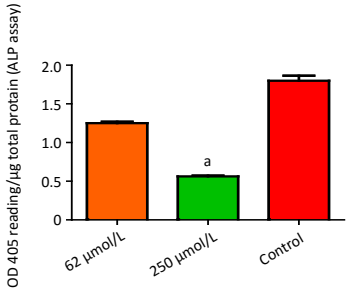
Note: Cobalt-chromium particles are seen around the knee prosthesis

Figure 6 | General view around knee joint of mice in the cobalt ion group



Note: The Y-axis stands for the live/total cell ratio (%) which is measured by CytoTox96VR Non-Ratio Cytotoxicity Assay. The left side of each group was the experimental control group. $^{a}P < 0.05$, *vs.* control group; $^{b}P < 0.05$, *vs.* 62 μmol/L group

Figure 2 | MTT assay to estimate the proliferation of MC3T3-E1 cells treated by Co^{2+} via serial concentrations



Note: The Y-axis stands for the optical density (OD) value of the experimental groups standardized by the total protein concentration, while the control group had no ionic stimulating cells. $^{a}P < 0.05$, *vs.* control group

Figure 4 | Effect of the concentration of Co^{2+} on the expression of alkaline phosphatase (ALP) in preosteoblasts

MicroCT evaluation results (*in vivo*)

MicroCT scans showed that mice in the stable control group had good fixation and no displacement, bone ingrowth around the titanium nail could be seen obviously. There was visible bone resorption around the titanium nail in the cobalt ion group (**Figure 7**), and the percentage of changes of bone mineral density before and after treatment was significantly lower than that in the loose control group and the stable control group ($P < 0.05$; **Figure 8**).

Stability evaluation of titanium nail prosthesis-nail pulling test (*in vivo*)

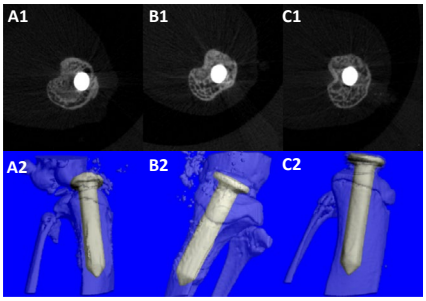
The blade holding the distal tibia of the mice which were fixed well by bone cement was able to withstand at least 100 Newtons (N) tension. In the stable control group, the maximum shear force needed at the moment of pullout was (11.58±2.47) N and (10.92±2.44) N in the loose control group, while in the cobalt ion group, the pullout force of the titanium nail was further reduced to (5.93±1.91) N. There were significant differences between the experimental group and the control group ($P < 0.05$; **Figure 9**).

Observation of histomorphology (*in vivo*)

Hematoxylin-eosin staining showed that there was obvious bone ingrowth and few inflammatory cells infiltration around the titanium nail in the stable control group. There was obvious inflammatory reaction membrane formation and a large number of inflammatory cell infiltration around the prosthesis in the cobalt ion group (**Figure 10A–C**). The comparison of periprosthetic membrane thickness between the above groups was calculated by computer image analysis software. The thickness of inflammatory reaction membrane around prosthesis in cobalt ion group was significantly thicker than that other two control groups ($P < 0.05$; **Figure 10D**). Differentiation and quantity of osteoclasts at the titanium nail prosthesis-bone interface were evaluated by TRAP staining, which showed that the degree of them in the surrounding tissues of the cobalt ion group was significantly higher than that of the control group ($P < 0.05$; **Figure 11**).

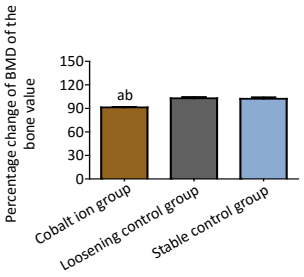
Immunohistochemical assessments (*in vivo*)

The expression of the positive-staining of TNF-α among groups is shown in the **Figure 12A–C**. There was almost no expression of



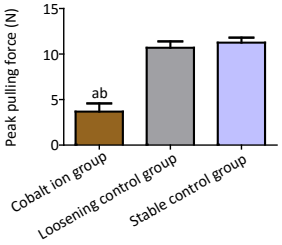
Note: (A1, A2) Obvious bone formation around the titanium nail in the stable control group. (B1, B2) Some bone formation in the loosening control group. (C1, C2) Bone absorption in the cobalt ion group

Figure 7 | Imaging data of postoperative MicroCT examination



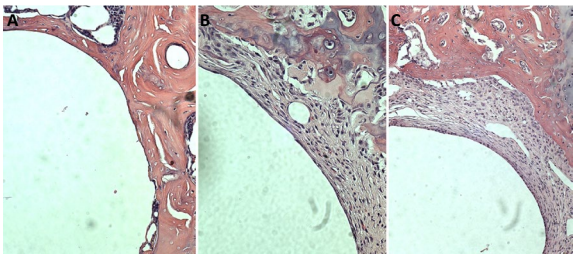
Note: The Y-axis stands for the percentage change of periprosthetic BMD in three groups (n=6). Data are measured by the evaluation program V6.5-1 software. [a]P < 0.05, *vs*. stable control group; [b]P < 0.05, *vs*. loosening control group

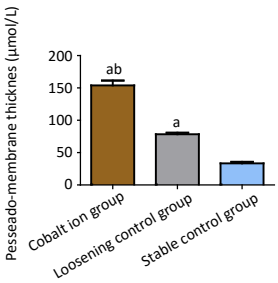
Figure 8 | Plot summarizes the percentage change of periprosthetic bone mineral density (BMD) in three groups



Note: The Y-axis stands for peak pulling force (N) which was analyzed and processed by the Bose WinTest® software. [a]P < 0.05, *vs*. stable control group; [b]P < 0.05, *vs*. loosening control group

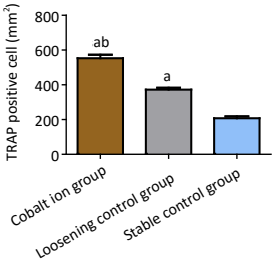
Figure 9 | Results of nail pull-out after knee joint amputation





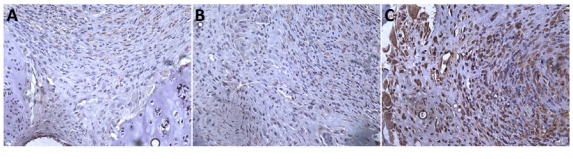
Note: (A-C) Hematoxylin-eosin appearances of pin-implanted tibia at 5 weeks following different treatments (×100). (A) Stable control group; (B) loosening control group; (C) cobalt ion group. (D) Comparison of periprosthetic membrane thickness in response to different groups. [a]P < 0.05, *vs*. stable control group; [b]P < 0.05, *vs*. loosening control group

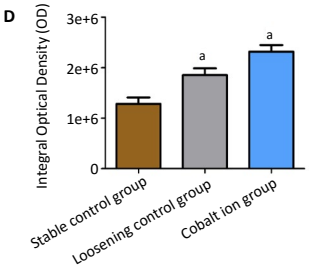
Figure 10 | Pseudo-membrane thickness by hematoxylin-eosin staining



Note: The quantity of TRAP positive cells was measured by Image-Pro Plus software in six different fields and expressed as ALP positive cells/mm^2. [a]P < 0.05, *vs*. stable control group; [b]P < 0.05, *vs*. loosening control group

Figure 11 | Comparison of quantity of TRAP positive cells in three groups





Note: (A-C) The representative images (all 200× magnification) of immunohistochemical staining against TNF-α among groups. (A) Stable control group; (B) loosening control group; (C) cobalt ion group; (D) quantification of the immunostaining for production of TNF-α chemokines on xenograft sections among groups measured by a computerized image analysis system. [a]P < 0.05, *vs*. stable control group

Figure 12 | Data of immunohistochemical staining against tumor necrosis factor alpha (TNF-α) among groups

TNF-α in the stable control group, but the cobalt ion group was the most obvious. All staining conditions were summarized, and it was concluded that the cobalt ion group and loosening control group were significantly higher than the stable control group in staining (P < 0.05; **Figure 12D**).

DISCUSSION

Prosthetic joint replacement is an effective treatment for end-stage osteoarticular diseases to relieve the pain caused by joint diseases, promote the function of joints to a certain extent and improve the quality of life[22]. Aseptic loosening of prostheses which still the most common complication after prosthetic joint replacement is related to bone loss around prostheses. NAKAMURA et al.[23] and other scholars believe that the process of osteolysis is closely related to the inflammatory reaction caused by metal particles. Previous studies have reported the effects of different wear debris, including Co-Cr particles, polymethyl methacrylate, ultra-high molecular weight polyethylene on BMSCs, preosteoblasts and mature osteoblasts[5, 24], but the effect of the ionic morphology of wear particles on these cells have barely mentioned. We used MC3T3-E1 cells to detect the effects of Co^{2+} on chemokine expression, osteoclast formation and osteoblasts formation during inflammatory reaction.

Preosteoblasts are the intermediate form of bone marrow mesenchymal stem cells differentiating into mature osteoblasts, which are abundant in bone marrow stroma and easily affected by wear particles in the process of transformation to osteoblasts. Previous studies have used MC3T3-E1 cells as preosteoblast models[25-26].

In vitro, to better study the biological effects of metal ions on the preosteoblasts, we established the optimal concentration of bivalent cobalt ions which is 62–1 000 μmol/L for the first time[19, 27]. MTT cell proliferation assay after 72 hours of co-culture with different concentrations of Co^{2+} showed the degree of proliferation of MC3T3-E1 cells would be significantly inhibited compared with the control group When Co^{2+} ≥ 125 μmol/L. LDH which exists in almost all tissues and cells plays an important role in the process of energy metabolism and transformation, and cell damage caused by various factors can cause LDH overflow

and increase the activity of it. Our experiment showed obvious toxicity when Co^{2+} at 1 000 μmol/L, and slight toxicity at 500 μmol/L. The above data showed that high concentration of bivalent cobalt ions in the humoral microenvironment produced a large number of toxic reactions to the preosteoblasts, inhibiting their proliferation and leading to their apoptosis. The inhibition of ALP expression which predicts the degree of maturation of preosteoblasts into osteoblasts indicates that the function of preosteoblasts is damaged. Our study suggests that the concentrations of Co^{2+} (250 μmol/L) can inhibit the expression of ALP compared with the control group. The above studies demonstrated that Co^{2+} can damage the function of preosteoblasts, osteoblasts and osteogenesis.

Chemokines play an important role in the process of inflammation. MCP-1 as a potent chemokine can promote the differentiation and activation of osteoclasts[28]. In our experiment, Co^{2+} of high concentration (500 μmol/L) can promote the expression of MCP-1, which indicates that Co^{2+} is easier to recruit monocytes/macrophages and osteoclasts. Previous studies have shown that pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 are usually secreted in wear particles-mediated osteolysis, which accelerates osteolytic effect of osteoclasts[29]. DALAL et al.[30] believe that cobalt-chromium particles of any type promote the release of inflammatory factors, such as TNF-α, IL-1β, IL-8 and IL-6 by osteoblasts, fibroblasts, and monocytes/macrophages. We studied the effects of Co^{2+} on TNF-α and IL-6 genes and the results showed that Co^{2+} at different concentrations promoted the release of inflammatory factors which was consistent with the IHC results *in vivo*. The system of RANKL/RANK/OPG is closely related to the differentiation of osteoclasts. Osteoblasts and bone marrow stromal cells express a certain amount of RANKL in physiological state and secrete a corresponding amount of OPG to maintain a balance between osteogenesis and osteolysis[31].

Our study showed that RANKL was significantly up-regulated, while OPG was significantly inhibited when MC3T3-E1 cells were co-cultured with Co^{2+}, demonstrating that Co^{2+} could promote osteoclast formation and lead to osteolysis *in vitro*. The low RANKL/OPG ratio may confirm the chemical staining results of $TRAP^{+}$ cells in the bivalent cobalt group and another function of preosteoblasts is to regulate osteoclast production by adjusting the ratio of RANKL/OPG[32], which can also provide a solution for the treatment of aseptic prosthesis loosening in the future. The effect of NFATc1 on osteoblasts is still controversial but our experiment showed that two different concentrations of Co^{2+} were likely to up-regulate the effect of NFATc1 on osteoblasts. Signaling proteins such as Runx2, Lrp5 and Osx also participate in the transformation of preosteoblasts into osteoblasts. In the current study, the expression of Osx gene was inhibited by different concentrations of Co^{2+} and the gene expression of Lrp5 and Runx2 was reduced by high concentrations of Co^{2+} (500 μmol/L). It further indicated that Co^{2+} could significantly damage the maturation and differentiation of osteoblasts from preosteoblasts, resulting in the reduction of the number of osteoblasts and the impairment of their functions. However, it should be noted that low concentrations of Co^{2+} (62 μmol/L) promoted the expression of Lrp5 and Runx2 genes, which need further study to explain. Through the RT-PCR data, the effects of bivalent cobalt ions on the differentiation, maturation and regulation of osteoclasts were further explained at the gene level, and the molecular biological basis of aseptic loosening of some artificial prostheses was revealed to a certain extent.

In order to study the mechanism and treatment of aseptic prosthesis loosening, many researchers have explored and established many animal models to simulate this pathological process[33-34]. However, these animal models are all short-term osteolysis models and lack the corresponding mechanical stress stimulation, so they cannot really simulate the biological process of prosthesis loosening. We constructed a long-term aseptic loosening animal model of joint prosthesis by implanting titanium nails in the proximal tibia of mice (Pin-model). Considering that wear particles are essential for aseptic prosthesis loosening, we injected cobalt and chromium particles suspension into the tibial bone marrow cavity of mice in the loosening control group and cobalt ion group before the implantation of titanium nails. MicroCT scan and three-dimensional reconstruction can compare the changes of the position of the titanium nail and the bone mineral density around the titanium nail after operation and 5 weeks after operation. The pull-out test can evaluate the strength of the prosthesis-bone interface and also reflect the stability of the implant. Our experimental results showed that the bone mineral density around the prosthesis in the cobalt ion group was significantly lower than that the control group, and the average force required for pulling out the nails was 5.93 N, which was also significantly lower than that the control group. Histological staining showed that a large number of inflammatory cells infiltrated around the prosthesis in the cobalt ion group, and the amount of mature osteoclasts was much higher than that of the control group. These *vitro* experiments have confirmed that Co^{2+} can inhibit the transformation of preosteoblasts to osteoblasts, affect bone formation and promote osteoclast formation leading to bone dissolution.

The deficiency existing in this experiment is mainly the experiment selects the cobalt ion concentration in the range of 62–1 000 μmol/L, we can just only estimate the ion concentration of instant around the prosthesis roughly, and whether other ions as chromium ion, titanium, molybdenum ions and aluminum ions also involved is not known, it will be our next research direction. Another limitation is that the body weight, life span and exercise intensity of mice are still quite different from that of humans, so this experimental animal model still cannot truly simulate the decades-long clinical prosthesis loosening process, which still needs to be further improved.

In this study, *in vivo* and *in vitro* experiments confirmed that preosteoblasts play an important role in aseptic loosening of prosthesis. Co^{2+} can inhibit the function of preosteoblasts to a certain extent, and promote the formation of osteoclasts and bone resorption by promoting the release of cytokines such as MCP-1, TNF, IL-6 and RANKL. In a word, Co^{2+} participates in the transformation of preosteoblasts and regulates their functions but the response of preosteoblasts to different concentrations of Co^{2+} during aseptic loosening of prosthesis may be different.

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假体无菌性松动过程中 Co^{2+} 对成骨前体细胞的生物学反应

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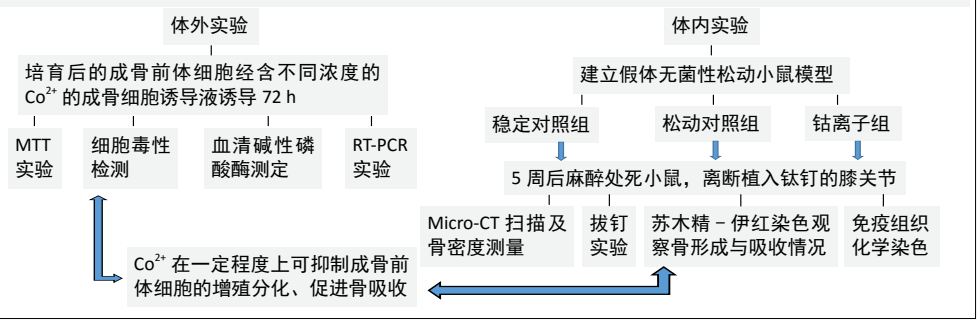
文章快速阅读：

文章特点—

△通过体外实验首先探讨不同浓度的二价钴离子对成骨前体细胞的生长、分化成熟、参与炎性反应及破骨细胞调控等所产生的不同影响；

△构建小鼠膝关节假体无菌性松动模型，通过体内实验进一步验证二价钴离子 (Co^{2+}) 刺激的成骨细胞前体细胞对破骨细胞分化成熟的重要调控作用。



文题释义：

磨损颗粒：人工关节置换后假体关节的微动造成的摩擦可产生磨损颗粒，常见的磨损颗粒包括金属颗粒(钴铬钼、钛)、超高分子量聚乙烯颗粒(UHMWPE)、骨水泥颗粒(PMMA)及陶瓷颗粒等。

假体松动：包括假体感染性松动和无菌性松动。感染性松动是由于术中或术后感染所致，机制明确。无菌性松动机制较为复杂，目前认为是由于磨损颗粒引起的骨溶解、吸收导致假体周围骨性支撑力学结构性能降低所致。

摘要

背景：假体无菌性松动是人工关节置换后的主要并发症。目前金属离子已被证实参与人工假体无菌性松动的过程，如何控制和减缓假体松动成为当前研究的热点。

目的：观察在假体松动过程中，不同浓度的二价钴离子(Co^{2+})刺激成骨前体细胞的生物学反应。

方法：①体外实验：小鼠成骨前体细胞(MC3T3-E1)分别与含不同浓度Co^{2+}的成骨细胞诱导液共同培养72 h，诱导为成骨细胞；应用MTT法检测细胞的增殖能力；通过测定乳酸脱氢酶活性来反映不同浓度Co^{2+}的细胞毒性；测定血清碱性磷酸酶蛋白浓度来检测成骨前体细胞向成骨细胞转化的能力；应用RT-PCR测定相关因子mRNA表达；②体内实验：将小鼠分为3组，稳定对照组将钛钉置入小鼠的胫骨近端、松动对照组置入钛钉和钴铬颗粒、钴离子组置入钛钉和钴铬颗粒并注入经过钴离子刺激的成骨前体细胞，术后立即使用 MicroCT 进行假体周围骨密度测定，5周后再次行假体周围骨密度测定，麻醉处死小鼠并离断患侧膝关节进行拔钉实验，并将拔钉后的组织进行苏木精-伊红染色。通过拔钉力量来测定假体松动程度；通过假体与骨界面之间膜的厚度来反映炎症反应的严重程度；通过抗酒石酸酸性磷酸酶染色观察假体周围组织中破骨细胞数量。

结果与结论：①体外实验结果：当Co^{2+}浓度升高时，成骨前体细胞的增殖会受到抑制；Co^{2+}对成骨前体细胞表达血清碱性磷酸酶具有显著的抑制作用；Co^{2+}可促进单核细胞趋化蛋白1、肿瘤坏死因子α、白细胞介素6、核因子κB 受体活化因子配基(RANKL)、活化T细胞核因子c1(NFATc1) mRNA表达，抑制骨保护素及成骨细胞特异性转录因子mRNA表达；低浓度Co^{2+}(62 μmol/L)促进低密度脂蛋白受体相关蛋白(Lrp-5)和Runx2 mRNA表达，高浓度Co^{2+}(500 μmol/L)抑制其表达；②体内实验结果：MicroCT 扫描显示钴离子组小鼠骨密度值最低($P < 0.05$)；钴离子组中，拔钉实验所需要的剪切力较对照组明显降低($P < 0.05$)；苏木精-伊红染色染色显示，钴离子组假体周围炎性反应膜的形成，Co^{2+}刺激的成体前体细胞可能会加重假体周围炎症反应；③结果证实，成骨细胞在假体无菌性松动中可发挥重要作用，Co^{2+}刺激的成骨细胞前体细胞对破骨细胞分化成熟的具有重要调控作用。

关键词：成骨前体细胞；金属离子；磨损颗粒；无菌性松动；炎症因子；破骨细胞；肿瘤坏死因子；白细胞介素6；碱性磷酸酶；组织工程

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