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Particulate and ion forms of Cobalt-Chromium challenged preosteoblasts promote osteoclastogenesis and osteolysis in a murine model of prosthesis failure

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Abstract

This study investigated the interactive behavior of the particulate and ion forms of Cobalt-Chromium (Co-Cr) alloy challenged preosteoblasts during the process of prosthetic implant loosening. Preosteoblasts were challenged with Co-Cr particles or Co(II) ions for 72 hours, followed by the proliferation and PCR assays. For *in vivo* test, a titanium-pin was implanted into proximal tibia of SCID mice to mimic knee replacement. Co-Cr particles or Co(II) ion challenged preosteoblasts (5×10^5) were intra-articularly injected into the implanted knee. The animals were sacrificed 5 weeks post-op, and the prosthetic knees were harvested for biomechanical pin-pullout testing, histological evaluations, and microCT assessment. *In vitro* study suggested that Co-Cr particles and Co(II) ions significantly suppressed the proliferation of preosteoblasts in a dose-dependent manner. RT-PCR data on the challenged cells indicated over-expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and inhibited osteoprotegerin (OPG) gene expression. Introduction of the differently challenged preosteoblasts to the pin-implant mouse model resulted in reduced implant interfacial shear strength, thicker peri-implant soft-tissue formation, more TRAP+ cells, lower bone mineral density and bone volume fraction. In conclusion, both Co-Cr particles and Co(II) ions interfered with the growth, maturation and functions of preosteoblasts, and provides evidence that the metal ions as well play an important role in effecting preosteoblasts in the pathogenesis of aseptic loosening.

INTRODUCTION

Since 1990, the prevalence of primary total hip replacement (THA) cases have increased by 50%, and the knee arthroplasties (TKA) are almost tripled. Meanwhile, the revision surgery of total joint arthroplasties has jumped by 17.5%.¹ Some studies reported that 56% of failure

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S-YY, SY and KZ designed the study. SY and S-YY formulated the manuscript. SY, JJ and BJ acquired and processed the data. All authors discussed the results and interpretation of data, and approved the manuscript.

cases of metal-on-metal (MOM) THA was because of aseptic acetabular loosening.² However, the mechanism of the prosthetic component loosening has still been debated. As MOM articulations are implanted with the assumption of better stability and lower wear rates compared to the metal-on-polyethylene prosthesis, consequent wear debris produced at bearing surfaces has been proved to play important roles in progress of aseptic loosening.^{3–5} After all, it appears inevitable to generate and release of the particulate and ion forms of metal debris on MOM prostheses due to bio-corrosion and articulation. Recent studies have suggested that the patients with MOM prosthesis suffered increased cobalt(II) and chromium(III) ion levels in their blood and plasma at short or long follow-ups,^{6–9} other studies manifested that adverse biological reactions including local soft tissue toxicity, bone loss, inflammation could be associated with high metal ion concentrations.^{10,11} However, the relationship between metal ions and aseptic loosening remains unclear.

Osteoblasts and their precursor cells (preosteoblasts) are naturally presence at the prosthesis site and keep close contact with the prosthetic component. Investigations showed that preosteoblasts played a crucial role to balance of bone formation and resorption at the interface between bone and prosthesis. Some studies reported that osteoblasts or their precursor cells responded to wear debris challenges resulting in increased osteoclastogenesis and bone resorption.^{4,12–14} Although there are studies investigating the influences of Ti(IV), Co(II) and Cr(III) on lymphocyte, monocyte, and osteoblast,^{11,15–18} there is no direct evidence whether the metal ions can result in aseptic loosening at *in vivo* pre-clinical settings. We hypothesize that both particulate and ion forms of Co-Cr alloy will challenge preosteoblasts and play equally important roles in regulating the balance of the bone turn-over and the osteoclastogenesis. The current study intends to investigate the interactive behaviors of preosteoblastic (MC3T3-E1) cells challenged by Co-Cr particles and Co(II) ions in a murine knee-prosthesis failure model.

MATERIALS AND METHODS

Particulate and ion forms of Co-Cr alloy

Co-Cr alloy particles (the mean particle size 5.7 μm , range 0.5–20 μm) were the gift of Dr. Jack Parr (Wright Medical, Memphis, TN). The particles were washed by 70% ethanol solution and confirmed endotoxin-free using the Limulus assay before use (Endosafe; Charles Rivers, Charlestown, SC). Co(II) chloride hexahydrate (Catalog Number: C8861) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). The ion form of Co(II) was dissolved in culture medium and sterilized by a 0.2- μm pore size membrane filtration (ThermoFisher, Rochester NY).

Particles and ion forms of Co-Cr on preosteoblast differentiation *in vitro*

MC3T3-E1 cells (CRL-2594, LOT: 60186879, ATCC, Manassas, VA, USA) were cultured in an Alpha Minimum Essential Medium(α -MEM) (GIBCO) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen) at 37% and 5% CO₂ atmosphere. For differentiation to osteoblasts, 10 mM β -glycerol phosphate (Sigma-Aldrich, St. Louis, MO), 50 $\mu\text{g/ml}$ L-

ascorbic acid (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich) were supplied into α -MEM.¹⁹

Cell proliferation in response to biomaterial challenge was evaluated by MTT assay, 5×10^4 MC3T3-E1 were dispensed into each well of the 96-well plates in the presence and absence of the Co-Cr particles (0.3 or 2.5 mg/ml) and ion Co(II) (62 or 500 μ M), respectively, for 72 hours. MTT assay was performed as previously described.¹⁴

RT-PCR was performed as detailed previously to evaluate the gene expression (MCP-1, TNF- α , IL-6, RANKL, OPG and Runx2) of MC3T3-E1 challenged by Co-Cr particle and ion Co(II).⁴ 1×10^5 cells per well were seeded in 12-well plates, which were cultured under osteogenic induction medium for 72 hours in the absence and presence of Co-Cr particle (0.3, 2.5 mg/mL) and ion Co(II) (62, 500 μ M). Briefly, total RNA from the cell homogenates was extracted using a commercial kit (Tel-Test, Friendswood, TX). Complementary DNA (cDNA) was reverse transcribed from 0.5 μ g of total RNA in a 40 μ L reaction mixture containing 1 $\times$ PCR buffer, 5.5 mM MgCl₂, 500 μ M each deoxynucleotide triphosphates, 0.5 U/ μ L RNase inhibitor, 2.5 μ M random hexamers, and 1.25 U/ μ L reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT), using a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, 48°C for 25 minutes, and followed by 95°C for 5 minutes. Tested gene expression was amplified in a StepOnePlus® Real-Time PCR System (Applied Biosystems) in a reaction mixture of SYBR Green PCR Master Mix (Applied Biosystems), 2 μ L cDNA, and 400 nM target gene primer pairs. The fluorescent signals were recorded dynamically. The values of the threshold cycle (Ct) at which a substantial increase in reporter-dye signals was first detected were normalized against expression of a housekeeping gene (18S), and the comparative quantification of the target gene expressions among the biomaterial-challenged samples was calculated according to the formula given in the manufacturer's manual. The primer pairs for target genes were constructed by Sigma-Genosys (Woodlands, Texas).

Establishment of the murine joint prosthesis model

All the procedures on mice were approved by the Wichita State University Institutional Animal Care and Use Committee. Thirty-six female severe combined immunodeficient (SCID) mice (CB17-Prkdc SCID; Jackson Laboratory, Bar Harbor, Maine) at 3–4 weeks of age were quarantined in a pathogen-free environment for at least one week before experimentation. Titanium-alloy pins provided by Stryker Orthopedics (Mahwah, NJ) were 0.8 mm in diameter and 5 mm long with a flat large top of 1.2 mm diameter.²⁰ Briefly, after anesthetized with Xylazine and Ketamine, under aseptic condition a medial parapatellar incision of 5 mm was made on left hind limb to expose the proximal tibia condyle, a part of articulate cartilage astern of patellar ligament were removed using a #11 scalpel followed by reaming a intramedullary cavity at least 5 mm deep with a dental drill (diameter: 0.7 mm). The Ti-pin was pushed into the canal in a manner such that the surface of the pinhead was flush with the cartilaginous surface of the tibial plateau and contiguous with the joint surface. The knee was rinsed with sterile PBS containing antibiotics (100 U/ml penicillin + 100 mg/ml streptomycin), and the wound was sutured in layers. In order to mimic prosthetic wear, 30 mice received a 10 μ L of a Co-Cr particle suspension (4×10^4 particles)

pipetted into the tibia canal before insertion of pin implant during surgery.¹⁴ The rest 6 animals were stable pin implantation controls.

Intra-articular injection of particles and ions-challenged preosteoblasts

MC3T3-E1 cells at 6×10^4 cells per well of a 48-well plate were cultured with Co-Cr particles (2.5 mg/mL), or Co(II) (500 μ M) in osteoblast induction medium for 72 hours. Before collecting the cells, the plate wells were washed with PBS for three times to remove the particles and the ions. At 24 hours after the Ti-pin implantation surgery, the 30 mice with Co-Cr particles containing pin-implantation were randomly divided into 3 groups: (1) Co-Cr group: mice were given an intra-articular injection of 10 μ l medium containing 5×10^5 MC3T3-E1 cells challenged by Co-Cr particles (2.5 mg/mL) (n=12); (2) Co(II) group: mice were given an intra-articular injection of 10 μ l medium containing 5×10^5 MC3T3-E1 cells challenged by Co(II) ions (500 μ M) (n=12); (3) loosening group: mice were given an intra-articular injection of 10 μ l medium without cells (n=6). The fourth group was a stable group, 10 μ l medium was injected on mice with pin implantation without local particle challenges (n=6). All animals were sacrificed at 5 weeks after surgery, and the knee joints were collected for micro CT, biomechanical (pin-pullout), and histological evaluations.

Micro-computerized tomography (μ CT) scan

The μ CT scanning was performed on a SCANCO μ CT System (vivaCT 40, SCANCO Medical, Switzerland) immediately after the pin implantation and at sacrifice. The parameters of 114 μ A current, 70 KW voltages, 200ms exposure time, 400 projections and 30 μ m isotropic voxel size were set for the scans. The image of plain scan and reconstruction of the limbs and its bone mineral densities (BMD), bone volume fraction and total volume (BV and TV) were analyzed by μ CT evaluation program V6.5-1 software.

Interfacial shear strength test

After sacrifice, the mouse limbs with the implant intact were immediately removed by disarticulating the knee joint. The surface top the pin-implant and the tibia were prepared by removing all soft tissue, and the limb was cemented into a potting box as reported previously for the pin pullout test, utilized a Bose 3200 ElectroForce™ load frame (Bose Corporation, Eden Prairie, MN) which extracting the pin implant at a rate of 1mm/min. The extracting force was recorded as a function of time using Bose WinTest® software.

Histological Evaluation and Image Analysis

The peri-implant proximal tibiae were fixed in formalin, decalcified in 12% EDTA, and embedded in paraffin for routine histology process and hematoxylin and eosin (H&E) staining. A computerized image analysis system with the Image-Pro+ software was used to examine for evidence of inflammation, bone formation or resorption around pin and the thickness of the soft-tissue membrane at the bone-pin interface. Immunohistochemical (IHC) stains were proceeded to semi-quantify the expressions of TNF- α , RANKL and Runx2 as described previously.^{1,21} In addition, histochemical TRAP staining (Sigma-Aldrich, St Louis, MO, USA) on paraffin-sectioned prosthetic joints was performed to reveal osteoclastic cells.¹⁴ The positive stains were evaluated on six randomly selected microscopic

fields of the each peri-implant tissue section and recorded as integrated optical densities (IOD) or the counts of positive-stained cells.

Statistical analysis

Mean values $\pm$ SEM express the results. For statistical analysis of data, one-way analysis of variance was performed using the SPSS 19.0 software package (IBM SPSS, Chicago, IL). Significance was set at $p < 0.05$.

RESULTS

Effects of CoCr-particles and Co(II) ions on Cell morphology, proliferation and gene expression *in vitro*

During the proliferation and differentiation in complete osteoblast-induction medium, the naive osteoblastic cells exhibited polygonal morphology. However, Co(II)-challenged cells stretched out much more tentacles, some with bright area; while cells co-cultured with particulate forms of Co-Cr alloy showed appearance of spindle morphology with obvious particle-phagocytosis.

MTT assay suggested a significant suppression effect on cell proliferations in both particulate and ion forms of the metal challenged groups at dose dependent pattern (* $p < 0.05$, ** $p < 0.01$ Fig. 1). There is no significant difference between the groups of Co-Cr particle (2.5 mg/ml) and Co(II) ions (500 μ M) ($p = 0.07$).

Assessment of gene expression profiles revealed that there was a distinctly increased MCP-1 expression in cells with Co-Cr particles (2.5 mg/ml) and Co(II) ions (500 μ M), compared to control (* $p < 0.05$, ** $p < 0.01$). Furthermore, Co-Cr particles (2.5 mg/ml) provoked an average of 7-fold more MCP-1 expression than the Co(II) (500 μ M) group ($p < 0.01$). Expressions of pro-inflammatory cytokines such as TNF- α and IL-6 were significantly upregulated by Co-Cr particles and ions at various concentrations. Again, the effect of the particles (2.5mg/ml) on IL-6 expression appeared significantly more dramatic than Co(II) (500 μ M) ($p < 0.01$). All the experimental groups inhibited the expression of OPG gene in a dose-dependent manner, while the control group specimens exhibited high OPG gene expression. The ions at higher concentration (500 μ M) markedly promoted RANKL expression and inhibited the OPG gene expression that resulted in a significantly higher ratio of RANKL/OPG compared to the ratio from the Co-Cr particle group ($p < 0.01$). Runx2 mRNA levels were elevated in the group with low concentrated Co(II) ions (62 μ M, $p < 0.01$), but significantly diminished at high dose (500 μ M) ions as well as in Co-Cr particle groups ($p < 0.01$) (Fig. 2).

Surgery outcomes

All mice recovered from the implantation surgery and cell injection with no complications. There was no obvious difference among groups in their daily activity levels and weight changes. Also, there were no obvious structural differences of articulate surfaces among the four groups when examined by naked eyes at sacrifice.

Microcomputerized tomography (μ CT) scanning evaluation

The initial μ CT scan showed that all the metal implants were well placed in position. However, the scans at sacrifice suggested that there were many focal bone resorptions or pit erosions on specimens from challenged MC3T3-E1 cell groups compared to the those from the control group. Figure 3 illustrates the quantification of BMD and bone volume fraction (BV/TV) among groups. It suggested that both particulate or ion metal-challenged preosteoblast groups sustained more severe BMD loss at the peri-implant bone areas (** $p < 0.01$), although there was no significant difference between the particle and ion challenged groups ($p = 0.14$) (Fig.3-I). The analysis of bone volume fractions among groups exhibited similar result that injections of the challenged preosteoblasts further diminished the local bone fraction volumes (Fig.3-II).

Pullout test for implant stability

The cement was strong enough to fix distal limbs for pin pullout test. Figure 4 summarized the average forces used to dissociate the implant pins from the surrounding bones among groups. While $9.73\text{N} \pm 0.69$ was the peak force required on the loosening groups that was significantly less than the force to dissociate a stable implant ($12.38\text{N} \pm 0.86$); implants from Co(II)-challenged and Cr-Co particle-challenged MC3T3-E1 cell injection groups were worse in stability (Figure 4). There was no significant difference between the two experimental groups ($p = 0.17$).

Histological analyses and TRAP staining

Tightly stable fixation and little pseudo-membrane formation were noticed in the H&E stained peri-implant sections from the stable controls (Fig.5A). The rest three groups revealed dramatically different appearance. There was an obvious layer of peri-implant proinflammatory tissue present in all specimens, although sections from the particle challenged group exhibited significantly thicker soft tissue than those from the other two groups ($p < 0.05$, Fig.5B-E).

TRAP staining was performed to reveal the osteoclasts surrounding the pin implantation. Significantly more TRAP+ cells were noticed in the challenged cell-injection groups compared to the control group ($P < 0.01$), and the Co(II) group demonstrated a higher number of TRAP+ cells than the group with Cr-Co particle-treated cells ($p < 0.05$, Fig.5F-G).

Immunohistochemical assessments

The quantification of the positive-staining of TNF, RANKL and Runx2 among groups was expressed as IOD level differences. The data suggested that both metal particle and Co(II) ion challenged cell injection groups exhibited significantly stronger TNF, RANKL and Runx2 positive stains compared to the loosening controls except that there is no significant difference as to the Runx2 in the Co(II) ion group (Fig.6, * $p < 0.05$, ** $p < 0.01$). Although there was markedly higher Runx2 expression in Co-Cr group than the Co(II) group ($p < 0.01$).

DISCUSSION

Aseptic loosening has been realized one of the most common long-term complication following MOM total joint replacement.²² Studies have suggested that hypersensitivity and inflammatory responses to metals debris and metal ions may closely associated with this osteolytic process.^{17,23–25} However, there is still lack of information on effects of Co-Cr particle and Co(II) ions on osteoblastic progenitors while most studies focused on other types of cells (macrophages/monocytes, Mesenchymal stem cells, T-lymphocytes and osteoclasts) and other metal ions: nickel(II) and vanadium(V) and Titanium (IV).^{14,26,27}

It has been well recognized that wear and biocorrosion of Co-Cr alloy implant generate particulate and ion forms of debris.^{Schnabel, 1994 #46} Usually, metal ions can be released earlier than metal debris. While metal debris accumulated in the interface between prostheses may migrate to remote regions, the metal ions often readily dissolve into the body fluid and interact with cells in far wider areas that the exact biological mechanisms are still largely undisclosed. Although local levels of metal ions around prostheses appear inconclusive in the literature^{26,27}, it is speculated that a gradient of Co-Cr particles and Co(II) ions exists, surely with the highest levels directly adjacent to the bearing surface. Studies have found that about 7 μM of Co(II) and Cr(III) in the sera of patients with aseptic loosening and a maximum of 1.6 mM in the local skeletal tissue around failing prosthesis.^{28–30} One of the researches reported the higher metal ion concentrations around prostheses compared to urine, organs and serum levels by X-ray emission microanalysis.³¹ It is therefore estimated that local Co(II) levels may be as high as 0.50 – 1 mM, implying that the concentrations utilized in this study were within a clinically significant range.³² Further, our preliminary *in vitro* test³³ clearly suggested that particles (5 mg/ml) or ions (1mM) resulted in ubiquitous cell death compared to debris-free controls, while particles < 0.15 mg/ml or ions < 32 μM did not show any influence on preosteoblast proliferation. We then determined to use particles concentrations at 0.3 and 2.5 mg/ml, in comparison with the metal ions at 62 and 500 μM as physiological and clinical relevant low and high concentrations for the current study.

Our laboratory has recently established a mouse model of knee implantation with a metal pin in proximal tibia to mimic human weight-bearing knee replacement that can sustain for up to 6 months without signs of loosening. The current study established the pin-implantation model in mice with metal particle and cell introductions to mimic aseptic implant loosening condition, and evaluated using bio-mechanical, μCT , and histological approaches. The implanted pin pullout test was performed to evaluate interfacial strength, which also reflects implant stability. It was required averagely 9.73 N to pull out of the pin from surrounding bone for mice in loosening group. Injection of stimulated preosteoblasts to the knees with metal particle challenged failing prosthesis resulted in significantly loosening effects; and 7.31 N and 6.24 N averaged forces were required to dissociate the pin implant in particle and ion challenged cell-introduction groups, respectively. The data suggested that the particle and ion challenged preosteoblasts lost their ability of bone formation. The results from μCT scans were consistent with pullout test. The prostheses with stimulated preosteoblasts resulted in bone resorption around the pins and dramatic decreases of local

BMD and bone volume fraction, which was also complemented by the increased quantity of TRAP+ cells.

It had been well-known that the generation of wear-debris at the interface played a crucial role in the formation of periprosthetic inflammatory tissue and many of them were engulfed by or in close contact with macrophages, MSCs, osteoblasts, lymphocytes and other inflammatory cells.³ Studies also reported that metal ion such as Ti(IV) can also be up-taken by lymphocytes.²⁷ Although both particulate debris and ions promoted preosteoblasts to upgrade inflammatory gene expressions such as MCP-1, TNF and IL-6; it appeared that the particles exhibited much more chemotactic ability than ions, exhibiting significantly higher expression of MCP-1 that may explain the thicker pseudo-membranes at bone-implant interface following particle-challenged cell injection. The data complements a previous report³⁴ that orthopedic implant cobalt-alloy particles resulted in greater inflammatory cytokines than titanium and zirconium alloy-based particles on human osteoblasts, fibroblasts and macrophages.

It is confirmed that preosteoblast challenged by wear debris resulted in increased osteoclastogenesis and bone degeneration.³⁵ In the current study, both particles and ions reduced the proliferation of preosteoblasts *in vitro* in a dose-dependent manner. Runx2 is responsible to drive the differentiation of preosteoblasts to immature osteoblasts, and to promote expression of several key downstream proteins that maintain osteoblast differentiation and bone matrix production. Therefore, expression of Runx2 is vital to the proliferation of osteoblasts and the bone formation. In the current study, both forms of the metal debris prohibited the Runx2 gene expression, especially in the higher concentrated Co(II) ions and particles groups. Interestingly, the low concentration of Co(II) ions (62 μ M) did show some stimulated effect on Runx2 expression, which may warrant further investigation in the future.

It is postulated that the particles and ions impair bone formation by reducing the quantity of the osteoblastic precursor and retarding osteoblastogenesis. RANKL/OPG ratio plays a critical role in the differentiation of osteoclast from macrophage or osteoclast precursor, which indirectly dictates the quantity of bone resorption and the kinetics of remodeling.¹ *In vitro* experimental data from this study clearly revealed that higher concentrated particulate and ion forms of the metal alloy significantly elevated RANKL expression, in addition to inhibited OPG expression (Figure 2). Further analysis of the RANKL/OPG ratio among groups suggested that Co(II) ions exhibited much higher ratio compared with that from CoCr particles group, which complemented the animal experimental results that significantly more TRAP+ cells were found in the peri-implant tissue with Co(II) ion-challenged cell injection group (Figure 5).

CONCLUSION

The current study suggested critical effects of preosteoblasts in the process of aseptic loosening. Both Co-Cr particles and Co(II) ions diminished cell proliferation at a certain extent. However, significantly more MCP-1 gene expression and thicker pseudo-membrane manifested that Co-Cr particles dominated the periprosthetic inflammation. In contrast,

Co(II) group exhibited increased TRAP+ stained osteoclastic cells, concomitant with significantly higher RANKL/OPG ratio. Overall, not only Co-Cr particles but also Co(II) ions participate in the regulation of differentiation and function of preosteoblasts, which in turn will promote osteoclastogenesis and bone resorption. However, preosteoblasts may respond differently to various forms of Co-Cr alloy debris during the process of aseptic loosening.

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FIGURE 1.

MTT assay to estimate the viability and proliferation of MC3T3-E1 treated by various concentrated particulate Co-Cr (0.3, 2.5mg/ml) and ion Co(II)(62, 500 µM) for 72 hours (*p < 0.05, **p < 0.01 compared to the control).

FIGURE 2.

Gene expression profiles among MC3T3-E1 cells groups in complete osteoblast-induction medium following variant particle and ions challenges (0.3 and 2.5 mg/mL, 62 and 500 µM). The data of Panel A is expressed as comparative gene copies against readings from naive cell control group (*p < 0.05, **p < 0.01). Panel B summarizes mRNA expression changes in comparison with naive cell control controls (comparative quantification of RT-PCR results, expressed as the percentage changes of the target mRNA expression compared to the mRNA copies of control as a '0' change).

FIGURE 3.

The plot summarizes the bone mineral density (BMD) (panel I) and bone fraction volume/total volume (BV/TV) (panel II) in four groups. The insert (A-C) illustrated that (A) and (B) show a typical stable titanium pin implanted for 5 weeks in the loosening group, (C): a microCT reconstructed 3-Disosurfaces image (* $p < 0.05$, ** $p < 0.01$ compared to the loosening control group)

FIGURE 4.

The plot shows the average of peak pulling force required to pull out the implants at 5 weeks after different treatments. The insert illustrates the examples of actual traces of the pulling forces to fail the pin implant from the surrounding bone (* $p<0.05$, ** $p<0.01$ compared to the loosening group).

FIGURE 5.

(A-D) represent the histological appearances of pin-implanted tibias at 5 weeks following different treatments (100x): (A) stable group; (B) loosening group; (C) Co(II) group; (D) Co-Cr group; (E) The comparison of periprosthetic membrane thickness in response to different treatments; (F) The image illustrates a TRAP stained peri-implant tissue section with purple stained osteoclastic cells (100x), the inserted image (200x) magnifies the area pointed by black arrow; (G) The comparison of quantity of TRAP positive cells in four groups (* $p < 0.05$, ** $p < 0.01$ compared to the loosening control group).

FIGURE 6.

Panels A-E illustrate representative images (all 100x magnification) of IHC staining against RANKL among groups of (A) stable group; (B) loosening group; (C) Co-Cr group; (D) Co(II) group; (E) Panel E summarizes the quantification of the IHC stains against RANKL, TNF, and Runx2 using a computerized image analysis system (*p<0.05, **p<0.01 compared to the loosening group).