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*Original Article*

# Therapeutic effects of OP-1 on metal wear particle induced osteoblasts injury *in vitro*

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**Abstract:** Aseptic lossening is a main reason for the revision of total joint arthroplasty. Metal-wear particles induced deregulation of bone resorption or formation has been considered as the major process of aseptic lossening. Osteogenic protein-1 (OP-1) can be used to improve bone formation. However, such effect is not clearly understood after the metal-wear particles injury. Here, we investigated the molecular mechanisms by which OP-1 regulates the activity of bone formation and anti-inflammatory after injury. Results showed that OP-1 increased cell viability and bone formation ability of impaired osteoblast cells at 72 hours after being injured by cobalt particles. Pathway analyses revealed that both mRNA and protein levels of Smad1 and Smad5 were significantly increased upon the treatment of OP-1 in the cell injury model. Similarly, runt-related transcription factor 2 (Runx2) was also significantly upregulated in the OP-1 treated cells. Moreover, treatment with OP-1 inhibited the secretion of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-18 in cobalt impaired cells. Collectively, these results suggest that OP-1 could inhibit cobalt particles induced cell injury by activating Smad1, Smad5, and Runx2, and such procedure is accompanied by anti-inflammatory reaction.

**Keywords:** Bone morphogenetic pritein-7, osteoblast formation, cobalt wear particles

## Introduction

Nowadays, total joint arthroplasty (TJA) is considered as a highly successful and cost-effective therapy for patients suffering from joint disease [1-3]. However, the revision rates of primary replacement of hip is about 1.29% and of knee is about 1.26% annually all over the world [4]. Also, the overall revision rate of TJA is increasing gradually in recent years [5], with a heavy economic burden about $8.5 billion on medicare system in the USA by 2015 [6]. Apart from the high economic burden of revision procedure, it can also increase the risk of post-operation infection because of the longer operative time and hospital stay compared with primary replacement [7]. Studies have found that aseptic loosening is the main cause for the revision in TJA. Recently, a literature showed that the incidence of aseptic loosening caused revision in total hip arthroplasty (THA), total knee arthroplasty (TKA), and total ankle arthroplasty (TAA) is about 55.2%, 29.8% and 38% respectively [8]. Both soluble and particulate debris derived from metal alley implants can induce local (and systemic) inflammation and result in decreased osteoblast formation and increased osteoclast activity [9].

Osteogenic protein-1 (OP-1) belongs to the transforming growth factor-β (TGF-β) superfamily, and is widely used in fracture and spinal fusion to promote bone formation [10, 11]. Beside its ability to promote bone formation, some other researchers report its ability to inhibit inflammatory responses [12, 13], and to regulate collagen synthesis of extracellular matrix (ECM) [14, 15]. Interestingly, some researchers have reported its anti-degeneration effect on cartilage *in vivo* [15-17]. A phase I safety and tolerability study of OP-1 in symptomatic knee osteoarthritis also have got posi-

tive results [18]. However, the mechanism underlying OP-1 on osteoarthritis remains speculative. Taking advantage of these effects, we hypothesize that OP-1 has the ability to decrease metal wear particles induced osteoblasts injury *in vitro*.

## Material and methods

### *MC3T3-E1 cell culture*

The osteoblastic cell line MC3T3-E1 from mouse was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in DMEM (Invitrogen, Carlsbad, USA), supplemented with 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/mLstreptomycin and 50 μg/mLascorbic acid. Cells were maintained in incubator with 5% $CO_2$ environment at 37°C. The medium was changed twice a week and the cells were sub-cultured using 0.05% trypsin with 0.01% EDTA.

### *Metal alloy wear particles*

Pure cobalt-alloy particles were kindly gifted from Nanjing University of technology, Nanjing, China. Sizes of them were less than 5 μm in diameter (mean 2.5 μm, SD ± 0.45 μm). Cobalt-alloy particles were washed three times in 70% ethanol and then sterilized in 70% ethanol overnight. Then, they were washed three times by sterile phosphate buffered saline (PBS) and resuspended in sterile PBS at 5 μg/mL, 10 μg/mLand 15 μg/mLrespectively. Use the Limulus Amoebocyte Lysate kit (LAL kit endpoint-QCL1000, Cambrex BioScience, Walkersville, MD, USA) to detect the endotoxin presence of alloy particles. Endotoxin was undetectable for all samples.

### *Cell proliferation assay*

Biotic influences of cobalt-alloy particles and treatment effect of BMP-7/OP-1 on osteoblasts were tested by cell counting kit-8 (CCK-8) (Shanghai RuianBioTechnologies Co., Ltd, Shanghai, China). Before treatment, cells were inoculated in a 24-well plate with a density about $2\times10^4$ cells per well. Then cells were cultured in 1.5 ml culture medium. After 24 hours, MC3T3-E1 cells were challenged with Co-alloy particles at doses of 0, 5, 10 and 15 μg/mL with or without 200 ng/mLOP-1. The 24-well plate was incubated in 5% $CO_2$ environment at 37°C. For each response group, the response time were divided into three group, 24 hours, 72 hours and 120 hours. MC3T3-E1 cell in control group was free of any interference. After treating cells with metal alloy particles and OP-1, cell proliferation was tested by CCK-8. The absorbance was measured at 450 nm by using the ThermoMK3 microplate reader (Thermo, Waltham, MA, USA).

### *Alizarin red staining*

Effects of metal alloy particles and OP-1 on cell mineralization ability were determined by alizarin red staining (Sigma, USA). Cells were treated as described above and stained following the manufacturer's protocol. A total of 0.1 g Alizarin Red S was added into 100 ml 0.1 M Tris-HCl buffer (pH 8.3) and stored in 4°C until use [19]. Cells were washed by PBS buffer for 3 times and fixed in 95% ethyl alcohol for 15 min and incubated with 1% Alizarin Red S solution for 10 min at RT. Experiments were repeated for 3 times and the results were consistent.

### *SDS-PAGE and western blot*

Cells were lysed with RIPA buffer. Protein samples were boiled at 95°C for 10 min and separated by SDS-polyacrylamide gel (10%) electrophoresis (Bio-Rad, USA), followed by Western blotting using a Nitrocellulose Transfer Membrane (Millipore, USA). After the transfer process, the membrane was probed with following antibodies: rabbit anti-pSmad 1 (Cell Signaling Technology, USA), rabbit anti-Smad 4 (Cell Signaling Technology, USA), Goat anti-Smad 5 (Santa Cruz, USA), rabbit anti-Smad 8 (Santa Cruz, USA), rabbit anti-Runx2 (Santa Cruz, USA) and mouse monoclonal anti-β-Actin (Sigma, USA) antibody, etc. Antibodies were diluted in 1×TBS and 0.05% Tween (Sigma, USA) at 4°C overnight. The secondary antibodies, peroxidase-labeled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, USA) was added for 2 hour at room temperature. β-actin and Tubulin were used as loading control. All experiments were repeated 3 times and data were consistent.

### *Quantitative RT-PCR*

After being challenged with cobalt alloy particles and OP-1, cell samples were re-culture in incubator for 72 hours. Total RNA was isolated

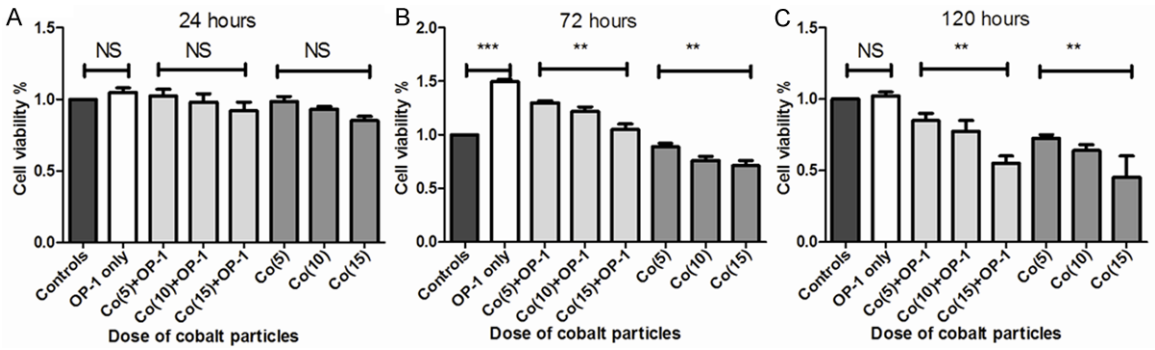


**Figure 1.** Cell viability of MC3T3-E1 cells at 24 hours (A), 72 hours (B) and 120 hours (C). Cells were treated with different doses of cobalt particles (Co) and OP-1 (200 ng/ml). NS, not significant. *P<0.05. **P<0.01.

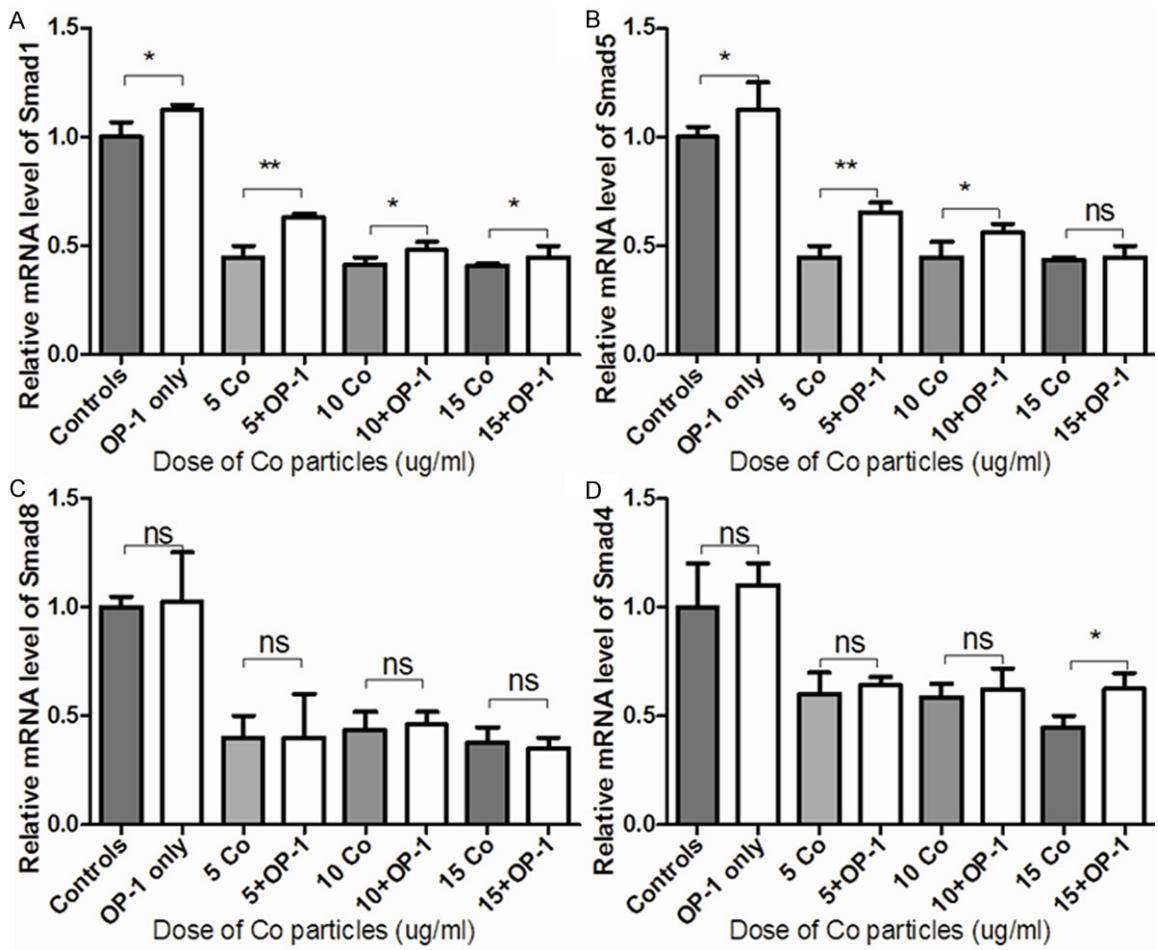


**Figure 2.** Relative mRNA levels of Smad1 (A), Smad5 (B), Smad8 (C), and Smad4 (D) in MC3T3-E1 cells treated with different doses of cobalt particles (5, 10, or 15 μg/mL) and OP-1 (200 ng/mL). NS, not significant. *P<0.05. **P<0.01. Data were adjusted to yield an arbitrary value of 1 for control templates using the (delta)(delta)Ct method.

from Osteoblasts by using Trizol method. Primers for selected genes were as follows: Smad1 (forward: 5'-CGTCCAACAATAAGAACCGC TTC-3'; reverse: 5'-GGCATTCCGCATACACCTCTC-3'); Smad4 (forward: 5'-ACAAGTAACGATG-CCTGTCTGAG-3'; reverse: 5'-AGCCACCTGAA-GTCGTCCAT-3'); Smad5 (forward: 5'-GAGAG-TCCAGTCTTACCTCCAGT-3'; reverse: 5'-TGCGG-

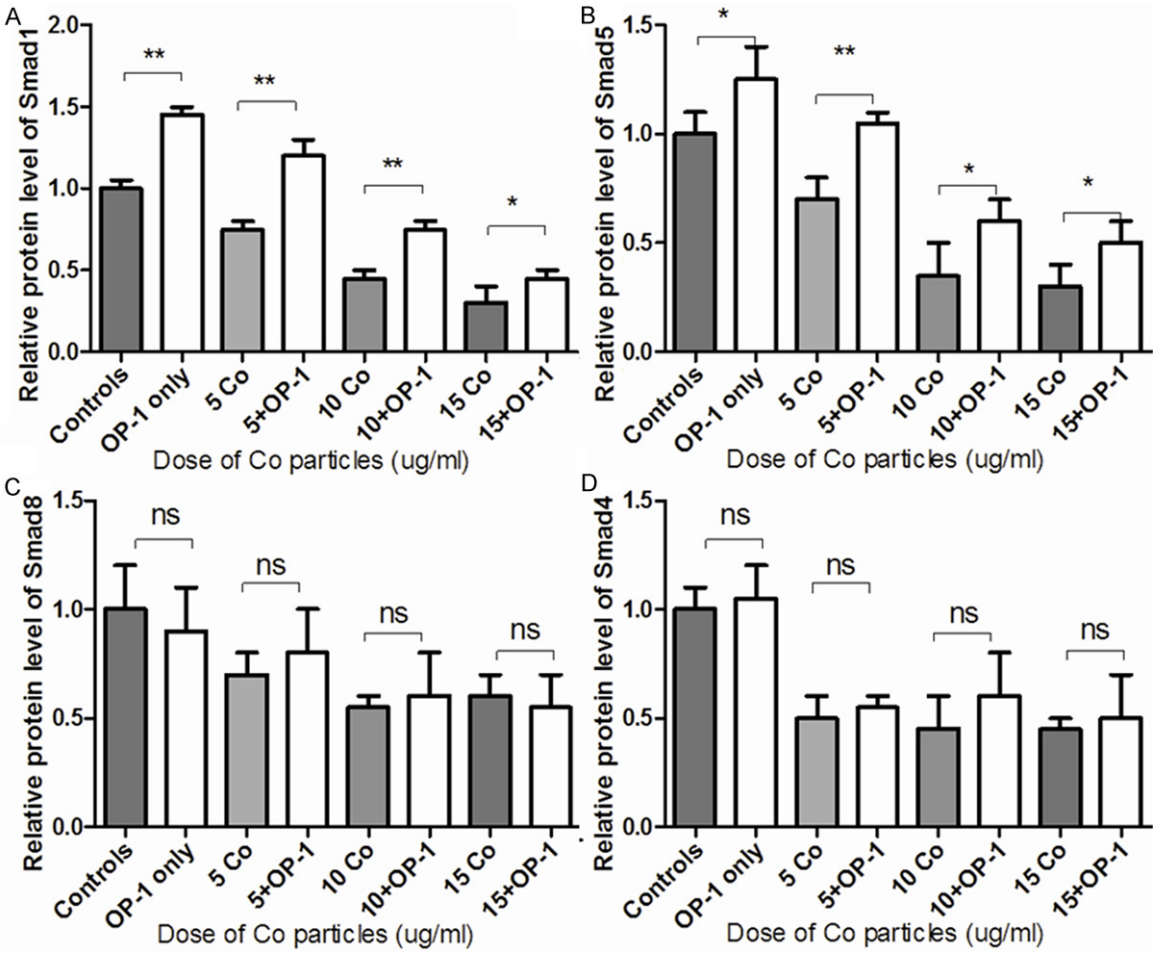


**Figure 3.** Relative protein levels of Smad1 (A), Smad5 (B), Smad8 (C), and Smad4 (D) in MC3T3-E1 cells treated with different doses of cobalt particles (5, 10, or 15 μg/mL) and OP-1 (200 ng/mL). β-actin were used as loading control. NS, not significant. *P<0.05. **P<0.01. Normalization: Smads/actin was first calculated and then pooled and normalized to 1.00.

TTCATTGTGGCTCAG-3'); Smad8 (forward: 5'-ACTGGTGTTCTGTTGCCTACTAC-3'; reverse: 5'-AAGATGCTGCTGTCGCTCAC-3'); Smurf1 (forward: 5'-GCAACATCGTCAGGTGGTTCT-3'; reverse: 5'-ATTGGCGTCTATCAGGTGAATGG-3'). Quantitative RT-PCR was performed at the condition of pre-degeneration at 94°C for 30 seconds, followed by 40 cycles denature at 94°C for 20 seconds, at 61°C for 30 seconds and at 72°C for 30 seconds and a 1 min terminal incubation at 72°C.

*Enzyme-linked immune sorbent assay of IL-6, TNF-α and IL-1β*

Levels of IL-6, TNF-α and IL-1β were examined by using ELISA kits (Mouse IL-6 DuoSet, R&D System, USA; Mouse TNF-α DuoSet, R&D System, USA; Mouse IL-1β/IL-1F2 DuoSet, R&D System, USA) according to the manufacturer's protocol.

*Statistical analyses*

Experimental variances between groups were performed by one-way ANOVA and unpaired-t test. Data were shown as mean ± SEM. *P* value<0.05 was considered as statistically significant. All statistical analyses were conducted by using SPSS 20.0 (SPSS, Chicago, IL).

## Results

*OP-1 improved the formation of injured osteoblasts*

MC3T3-E1 cells were challenged with Co-alloy particles at doses of 5, 10 and 15 μg/mL with or without 200 ng/mLOP-1. The statistical anal-

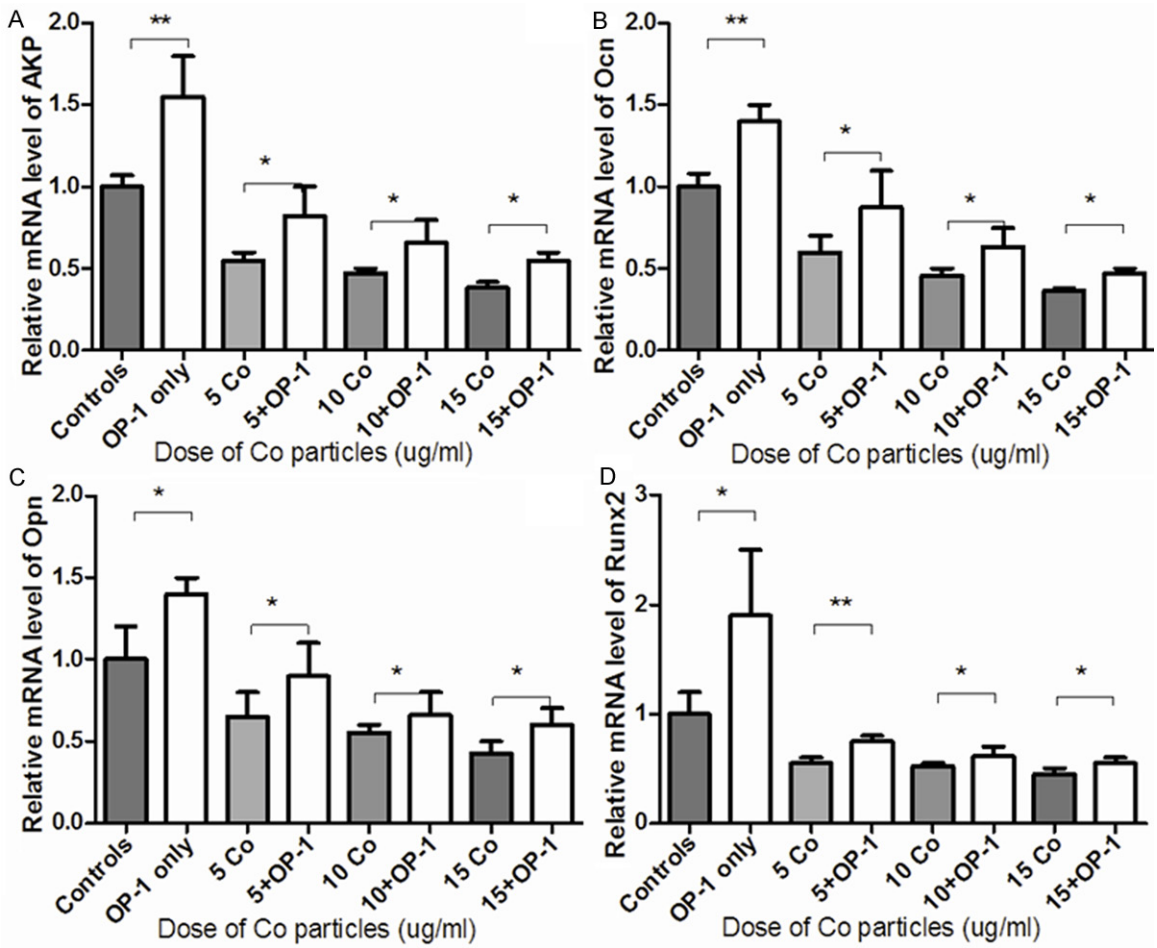


**Figure 4.** Relative mRNA levels of AKP, OCN, OPN and Runx2 in MC3T3-E1 cells treated with different doses of cobalt particles (5, 10, or 15 μg/mL) and OP-1 (200 ng/mL). NS, not significant. *P<0.05. **P<0.01. Data were adjusted to yield an arbitrary value of 1 for control templates using the (delta)(delta)Ct method.

yses were performed among the three groups (Co [5], Co [10], and Co [15]). Data showed that OP-1 treatment at 200 ng/mL did not significantly alter the cell viability at 24 hours (**Figure 1A**). However, it clearly increased the viability of cobalt particle treated cells at 72 hours (**Figure 1B**). Such effect of OP-1 on cell viability was disappeared at 120 hours (**Figure 1C**). These results indicated that cell viability of osteoblasts was decreased after being challenged with cobalt particles, and such decrease was in dose-dependent and time-dependent manners. To detect whether OP-1 can improve bone formation in cobalt particles induced osteoblast damage, we conducted experiment to test the mineralization ability of osteoblast cells by using Alizarin red staining. The staining result showed us that OP-1 could improve the bone formation ability after cobalt particles injury (data not shown). Thus, OP-1 could improve the cell viability and bone formation ability of injured osteoblasts.

*OP-1 upregulated post-injury osteoblast formation through Smad1, Smad5, and Runx2*

Studies have shown that Smads 1, 4, 5, and 8 could modulate the anabolic activity in osteoarthritic cartilage and play important roles in bone regeneration [20]. Here, we hypothesized that OP-1 may take participate in the process of osteoblast bone formation through the activation of Smad-dependent pathway. We first investigated mRNA levels of Smads in the cells treated with cobalt and OP-1. Data showed that cobalt particles clearly decreased mRNA expressions of Smad1, Smad4, Smad5, and Smad8 (**Figure 2**). Treatment with OP-1 significantly increased mRNA levels of Smad1 and

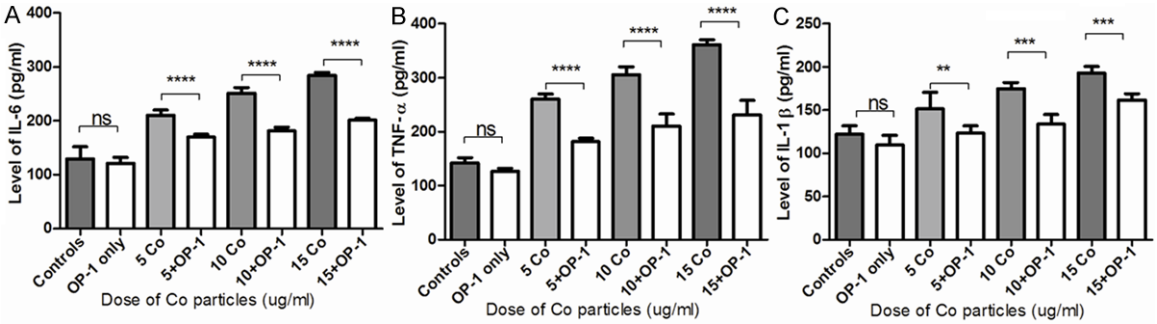


**Figure 5.** Levels of IL-6 (A), TNF-α (B) and IL-1β (C) in the supernatant of MC3T3-E1 cells treated with different doses of cobalt particles (5, 10, or 15 μg/mL) and OP-1 (200 ng/mL). NS, not significant. $^{*}$P<0.05. $^{**}$P<0.01. $^{***}$P<0.001. $^{****}$P<0.0001.

Smad5 in cells with different concentrations of cobalt (**Figure 2A** and **2B**), whereas mRNA expression of Smad4 was moderately elevated in OP-1 treated cells (**Figure 2D**). However, level of Smad8 remained unchanged upon the addition to OP-1 (**Figure 2C**). We further evaluated the protein levels of Smads in the OP-1 treated cells. Data showed that the protein expression of Smad1 and Smad5 but not Smad4 or 8 were clearly upregulated in OP-1 treated cells (**Figure 3**). In addition, alkaline phosphatase (AKP) has been shown to be correlated with the activation of bone formation. Osteocalcin (OCN), Runx2 and osteopontin (OPN) are critical in osteogenesis. They are thought to play a role in the body's metabolic regulation and bone mineralization. In many studies, OCN and OPN are used as preliminary biomarkers on the effectiveness of a given drug on bone formation. We evaluated mRNA levels of AKP, OCN, Runx2 and OPN in the cobalt-injured cells and found that mRNA expression of these factors significantly increased when the cells were treated with OP-1 (**Figure 4**). Protein levels of AKP, OCN, and OPN remained similar when adding OP-1, whereas protein expression of Runx2 was clearly upregulated in OP-1 treated cells (data not shown). These data indicate that Smad1, Smad5, and Runx2 could be critical pathways for the function of OP-1.

*OP-1 inhibited inflammatory responses in injured osteoblasts*

It has been reported that metal alloy particles has pro-inflammatory effects on osteoblast cells [21]. To detect whether OP-1 would influence levels of pro-inflammatory factors, we measured cytokine expression of the injured osteoblasts (**Figure 5**). After being exposed to OP-1 (200 ng/ml), the level of IL-6, TNF-α and IL-1β decreased significantly in cobalt injured osteoblasts (P<0.05). Thus, besides its effect on improving bone formation, OP-1 could also inhibit inflammatory response induced by cobalt particles *in vitro*.

## Discussion

A large number of patients suffer from the revision surgery of TJA, and the main reasons of revision are metal-alloy particle-induced osteolysis and local inflammation [21]. Considering the biological function of OP-1, we conducted this study and demonstrated that OP-1 could improve bone formation and inhibit inflammation response in cobalt-injured osteoblasts *in vitro*. In the cell viability experiments, we did not observe an immediate effect of OP-1 on cobalt-injured osteoblasts (**Figure 1A**). However, it greatly elevated the cell viability at 72 hours (**Figure 1B**). Also, by using Alizarin red staining, we found that OP-1 was able to repair impaired bone formation process (**Figure 2**), which was consistent with pervious data by other researchers [22].

SMADs are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream gene transcription. Smads1, 4, 5, and 8, are expressed in normal human articular chondrocytes in vivo and in vitro and down-regulation of these mediators could be responsible for the decrease of anabolic activity in osteoarthritic cartilage. Here, we hypothesized that OP-1 may take participate in the process of osteoblast bone formation

through the activation of Smad-dependent pathway [23]. Our data showed that treatment with OP-1 significantly increased mRNA levels of Smad1 and Smad5 (**Figure 2A** and **2B**), and moderately increased mRNA expression of Smad4 in cells with different concentrations of cobalt (**Figure 2D**). However, level of Smad8 remained unchanged when treatment with OP-1 (**Figure 2C**). Protein analyses confirmed that expression of Smad1 and Smad5 but not Smad4 or 8 were clearly upregulated in OP-1 treated cells (**Figure 3**). These results suggest that the effect of OP-1 on bone formation may be through Smad1 and 5.

Runx, a family of transcription factors, regulates several biological events, such as hematopoiesis, bone formation and homeostasis of gastric mucosa. Past research found that mice lacking the Runx2 gene showed complete loss of bone formation. Runx2 and BR-Smads (Smad1/5/8) physically interact with each other upon activation of BMP signal pathway. In addition, AKP has been shown to be correlated with the activation of bone formation. Smurf1, OCN and OPN are critical in osteogenesis. It is possible that OP-1 may function through these factors. In our study, mRNA levels of Runx2, AKP,OCN and OPN were significantly increased after OP-1 treatment (**Figure 4**). However, only the protein expression of Runx2 was elevated with OP-1 (data not shown), indicating that Runx2 may be another major pathway of OP-1 on bone formation.

In conclusion, our data showed that OP-1 could inhibit cobalt particles induced cell injury in osteoblast by activating Smad1, Smad5, and Runx2. Moreover, OP-1 could suppress cobalt particles induced local inflammation. These findings provide novel knowledge for improving the treatment of implant-induced osteolysis after TJA.

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## Disclosure of conflict of interest

None.

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