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Original Paper

Carbon Monoxide Releasing Molecule 3 Inhibits Osteoclastogenic Differentiation of RAW264.7 Cells by Heme Oxygenase-1

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Key Words
CORM-3 • RAW264.7 cell • Osteoclastogenic differentiation • HO-1

Abstract
Background/Aims: Increased osteoclastogenic differentiation may disrupt the balance of bone resorption and formation, giving rise to bone defective disease. The study aimed to investigate the influence of carbon monoxide releasing molecule 3 on osteoclastogenic differentiation of RAW264.7 cells, and explore the possible mechanism underlying the regulatory effect. ***Methods:*** Influence of CORM-3 on the proliferation of RAW264.7 cells was determined by CCK-8 assay. RAW264.7 cells were divided into four groups: Control group; Osteoclastogenic differentiation group, in which cells were induced osteoclastogenic differentiation in medium supplemented with 100μg/L RANKL and 50μg/L M-CSF; Degassed CORM-3-osteoclastogenic differentiation group, in which cells were pretreated with 200μmol/L degassed CORM-3 for 6hrs, and then induced osteoclastogenic differentiation; CORM-3-osteoclastogenic differentiation group, in which cells were pretreated with 200μmol/L CORM-3, and then induced osteoclastogenic differentiation. The mRNA and protein expression of RANK, TRAP, MMP-9, Cts-K and HO-1 of the cells during the osteoclastogenic differentiation was checked by RT-qPCR and Western blot. The induced osteoclasts were identified by TRAP staining. The HO-1 expression of the RAW264.7 cells was silenced by lentivirus transfection, and the expression of RANK, TRAP, MMP-9 and Cts-K was examined by RT-qPCR and Western blot. ***Results:*** CORM-3 promoted the proliferation of RAW264.7 cells at the concentration of 200μmol/L. Pretreatment with CORM-3, but not degassed CORM-3, significantly decreased the mRNA and protein expression of osteoclast-specific marker TRAP, RANK, MMP-9 and Cts-K induced by RANKL and M-CSF on day 5, 7 and 9 during the osteoclastogenic differentiation ($P<0.05$). After HO-1 was silenced by lentivirus transfection, the mRNA and protein expression of TRAP, RANK, MMP-9 and Cts-K in group with CORM-3 pretreatment maintained the same level as in osteoclastogenic differentiation group. ***Conclusion:*** CORM-3 inhibits osteoclastogenic

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differentiation of RAW264.7 cells via releasing CO. The inhibitory effect is mediated partially by HO-1 pathway. The results suggest the potential application of CORM-3 on some bone defective diseases.

Introduction

Bone homeostasis is maintained through the dynamic balance between osteoblastogenesis which gives rise to bone formation and osteoclastogenesis which results in bone resorption [1-4]. Osteoclasts are derived from hematopoietic precursors of the monocyte-macrophage lineage in response to the stimulation of proinflammatory cytokines or factors, such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), both are key drivers of osteoclastogenesis and osteoclastic bone resorption [5, 6]. In addition to inflammatory cytokines, growth factors and hormones can also disrupt the balance of osteoclast and osteoblast activity, resulting in pathological changes.

Bone-forming osteoblasts express RANKL on their surface in response to a variety of stimulating factors. RANKL directly engages a membrane receptor, RANK, on osteoclast precursors and mature osteoclasts to trigger multiple intracellular signaling cascades that stimulate osteoclast gene expression, development, function and survival [6-8]. RANKL/RANK signaling cascade regulates not only the maturation of osteoclast progenitors, but also the activity of the osteoclasts. RANKL/RANK is therefore regarded as a key system that regulates osteoclasts differentiation, maturation and bone resorption [9, 10]. There have been reported that any inflammatory bone diseases causing bone loss or bone defect are RANKL-dependent [11, 12].

RAW264.7 cells are mouse-derived osteoclast precursor cells, which was originally derived from the tumor caused by the Abelson mouse leukemia virus. Studies have shown that the bone resorption-related genes, such as Cathepsin K (Cts-K), significantly increased after RANKL stimulation in RAW264.7 cells [13]. This cell line has been widely used in the research on osteoclastogenic differentiation.

Carbon monoxide (CO), a byproduct of heme catalysis by hemeoxygenase, has long been regarded as a poisonous gas. However, recent studies have demonstrated CO is cytoprotective as it induces vasorelaxation [14, 15], inhibits cell apoptosis [16], suppresses inflammation [17], and protects organs against ischemia/reperfusion injury [18, 19]. CO releasing molecules (CORMs) are newly synthesized CO compounds, which are able to efficiently release the controlled amounts of CO *in vitro* and *in vivo* biological models under appropriate conditions [20]. In particular, CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)] is fully water-soluble, can rapidly liberate CO when dissolved in physiological solutions. In our previous study, we have shown that CORM-3 inhibits the expression of adhesion molecules on human gingival fibroblasts induced by inflammatory cytokines, and reduces the infiltration and adhesion of immunocompetent cells via releasing CO [21]. CORM-3 has been shown to prevent reoccurrence of sepsis, and reduce cecal ligation and puncture-induced liver injury [22, 23].

Although many beneficial effects of CORMs have been demonstrated, the effect of CORMs on osteoclastogenic differentiation remains unclear. In the present study, we used RAW264.7 cells as an *in vitro* model to investigate the effect of CORM-3 on osteoclastogenic differentiation, and the possible mechanism underlying the regulation.

Materials and Methods

Reagents and Antibodies

Fetal bovine serum and DMEM high glucose medium were purchased from Gibco BRL (Grand Island, NY, USA). CORM-3 was purchased from Sigma (St.Louis, MO, USA). Trizol, reverse transcription kit and PCR amplification kit were purchased from Invitrogen (Carlsbad, USA). RANKL and M-CSF were purchased from Peprotech (Suzhou, China). RIPA, Protease inhibitor and SDS-PAGE Gel Kit were purchased from Solarbio (Beijing, China). PVDF membrane, anti-RANK primary antibody, anti-TRAP primary antibody, anti-MMP-9 primary antibody and anti-Cathepsin K primary antibody were purchased from Abcam (Cambridge, MA, USA).

Culture of RAW264.7 cells

RAW264.7 cells were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, U.K.). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100units/ml penicillin and 0.1mg/ml streptomycin. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence.

Cell proliferation assay

RAW264.7 cells were seeded in 96-well plate at a density of 10^4cells/well and cultured in DMEM medium supplemented with 10% FBS for 24hrs at 37˚C. Subsequently, cells were incubated in the DMEM medium containing CORM-3 at concentration of 0μmol/L, 100μmol/L, 200μmol/L, 400μmol/L, and 800μmol/L, respectively. CORM-3 was freshly prepared prior to the experiment by dissolving the compound in distilled water. Following 24 hrs and 48hrs, cell proliferation was checked by CCK-8 Kit (Dojindo Molecular Technologies, Inc., Beijing, china). Briefly, 10 μl cell counting Kit-8 was added to each well and cells were incubated for a further 2hrs at 37˚C. Subsequently, absorbance at 450 nm [optical density (Od) 450 nm] was measured using a SPECTROstar Nano ultraviolet spectrophotometer (Spectro Analytical Instruments GmbH, Kleve, Germany). The experiment was repeated in triplicate.

Induction of osteoclastogenic differentiation

RAW264.7 cells were divided into 4 groups. Control group, in which cells were cultured in DMEM supplemented with 10% FBS; Osteoclastogenic differentiation group, in which cells were cultured in osteoclastogenic differentiation medium: DMEM supplemented with 10% FBS, 100μg/L RANKL and 50μg/L M-CSF; Degassed CORM-3-osteoclastogenic differentiation group, in which cells were pretreated with 200μmol/L degassed CORM-3 for 6hrs, and then the medium was completely replaced with osteoclastogenic differentiation medium; CORM-3-osteoclastogenic differentiation group, in which cells were pretreated with 200μmol/L CORM-3 for 6hrs, and then the medium was completely replaced with osteoclastogenic differentiation medium. The medium was changed every third day in all groups.

CORM-3 was freshly prepared as aforementioned. Degassed CORM-3 was produced by dissolving CORM-3 in distilled water and placing the solution in a vacuum device at 37˚C for 24hrs prior to the experiments. Degassed CORM-3 was used as a negative control to assess the direct involvement of CO in the pharmacological activity of CORM-3 [20].

For osteoclastogenic differentiation experiments, cells were seeded in 6-well plate at a density of $5*10^4$cells/well and cultured in the indicated medium. The mRNA and protein expression of osteoclast marker genes TRAP, RANK, MMP-9 and Cts-K were subsequently analyzed.

RNA isolation and RT-qPCR

Following 5, 7 and 9 days culture, total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. 1μg of the total RNA was reverse transcribed into complementary DNA with Prime Script RT Master Mix (Takara, Kusatsu, Japan) according to the manufacturer's instruction. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara, Kusatsu, Japan) on the Applied Biosystems ViiA™ 7 Real-Time PCR System. The specific primers used in the present study were as follows: RANK (forward primer): 5′-AGCATTATGAGCATCTGGGACGG-3′ and reverse primer: 5′-CAGCAAGCATTTATCTTCTTCATTCC-3′; TRAP (forward primer):

5′-TGGTCATTTCTTTGGGGCTTATCT-3′ and reverse primer: 5′-GCTACTT GCGGTTTCACTATGGA-3′; MMP-9 (forward primer): 5′-GATCCCCAGAGCGTCATTC-3′ and reverse primer: 5′-CCACCTTGTTCACCTCATTTTG-3′; Cts-K (forward primer): 5′-TGA CCACTGCCTTCCAATAC-3′ and reverse primer: 5′-CTCTGT ACCCTCTGCATTTAGC-3′; GAPDH (forward primer): 5′-GTATGACTC TACCCACGGCAAGT-3′ and reverse primer: 5′-TTC CCGTTGATGACCAGCTT-3′; The cycle threshold (Ct) obtained for target gene expression was normalized to GAPDH, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ methods. Real-time quantitative PCR was used to detect the mRNA expression of RANK, TRAP, MMP-9 and Cts-K genes on the 5th, 7th and 9th day. The qPCR thermocycling conditions were as follows: initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 60 sec and extension at 72°C for 45 sec. GAPDH served as the housekeeping gene for normalization.

Western blot analysis

Following 5, 7 and 9 days culture, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and then lysed with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with phosphatase inhibitor (Sangon Biotechnology, Shanghai, China) and PMSF (Roche Molecular Biochemicals, Mannheim, Germany). The protein concentration of each group was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Aliquots of total protein were separated by 10% sodium dodecyl sulfate polyacry lamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Labora -tories, Hercules, USA). Membranes were blocked in 5% nonfat milk for 1h at room temperature and subsequently incubated with primary antibodies: rabbit anti-RANK (Abcam, Cambridge, U.K.) (1:500 dilution); rabbit anti-TRAP (Abcam, Cambridge, U.K.) (1:500 dilution); rabbit anti-MMP-9 (Abcam Cambridge, U.K.) (1:600 dilution); rabbit anti-Cts-K (Abcam, Cambridge, U.K.) (1:600 dilution); rabbit anti-GAPDH (Abcam, Cambridge, U.K.) (1:600 dilution); overnight at 4°C, respectively. After three washes, membranes were incubated with horseradish peroxidase-(HRP)-conjugated goat anti-rabbit IgG for 1h, washed again with TBST, and subsequently visualized using West-Pico ECL kit (Pierce, Rockford, USA).

Construction and selection of HO-1-shRNA lentiviral expression vector

Four shRNAs based on HO-1 mRNA sequence were designed. The shRNA expression cassettes of the target sequence followed by the loop sequence (CTTCCTGTCAGA), reverse complement to it. These shRNA expression cassettes and their complementary strands were synthesized commercially (Sigma, Victoria, Australia) and annealed in the annealing buffer following a protocol previously described. Briefly, the resulting double-stranded oligo DNAs were cloned into pLenR-GPH at the EcoR I and BamH I of the MCS sites through ligation reactions. To check the insert, the plasmids were digested and then assessed by 2% agarose gel electrophoresis for the inserted fragment. Because the plasmid also contains an eGFP gene, the transduction of the lentiviral vector can be monitor by fluorescent microscope. HO-1-shRNA lentiviral expression vector was constructed including ShNC, Sh1, Sh2 and Sh3 lentivirus. Real-time fluorescence quantitative PCR and western blot lentivirus screening technology was applied to select the most ideal HO-1-shRNA lentiviral expression vector.

Statistical analysis

Data were obtained from at least three independent experiments. The significance of difference was assessed by one-way analysis of variance method. Data are presented as the mean ± standard deviation and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of CORM-3 on the proliferation of RAW264.7 cells

RAW264.7 cells were incubated with different concentrations of CORM-3 for 24hrs and 48hrs. Cell proliferation was assessed by a CCK-8 kit. As shown in Fig. 1, 200μM CORM-3 significantly promoted the proliferation of RAW264.7 cells ($P<0.05$). Notably, 800μM CORM-3 significantly inhibited the cell proliferation compared with the control ($P<0.05$).

Based on the results of the experiment, 200μM CORM-3 was determined as the working concentration for the following experiments.

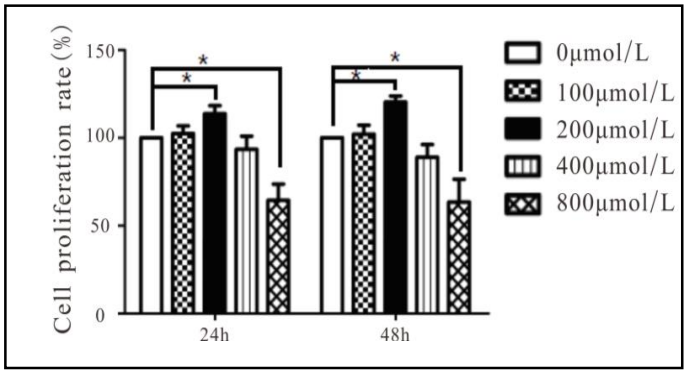

Fig. 1. Effects of CORM-3 on RAW264.7 cell proliferation. RAW264.7 cells were incubated with 0μM, 100μM, 200μM, 400μM, 800μM CORM-3 for 24hrs and 48hrs. Cell proliferation was assessed by a CCK-8 kit. Data are presented as the mean ± standard deviation (n=3). *: P<0.05 vs. control group.

Effect of CORM-3 on mRNA expression of TRAP, RANK, MMP-9 and Cts-K during osteoclastogenic differentiation

To investigate whether CORM-3 promoted the expression of osteoclast-specific genes, RAW264.7 cells were subjected to osteoclastogenic differentiation with or without CORM-3 pretreatment. The mRNA expression of TRAP, RANK, MMP-9 and Cts-K on the 5th, 7th and 9th day was assessed by RT-qPCR during the osteoclastogenic differentiation. Degassed CORM-3 was used to clarify the involvement of CO on the regulatory effect of CORM-3. As indicated in Fig. 2, the expression of TRAP, RANK, MMP-9 and Cts-K mRNA of the cells in osteoclastogenic differentiation group was significantly increased compared with the control group on day 5, 7 and 9 (P<0.05). Interestingly, the expression of TRAP and MMP-9 peaked on the 7th day, while the expression of RANK and Cts-K on the 5th day and 9th day, respectively.

CORM-3 pretreatment significantly decreased the mRNA expression of these four osteoclast-specific genes compared with cells without CORM-3 pretreatment at each time point (P<0.05). However, in degassed CORM-3-osteoclastogenic differentiation group, the level of TRAP, RANK, MMP-9 and Cts-K mRNA was parallel with that in osteoclastogenic differentiation group (P>0.05). The result that degassed CORM-3 failed to regulate the TRAP, RANK, MMP-9 and Cts-K mRNA expression suggested that the effect of CORM-3 was mediated by the release of CO.

Effect of CORM-3 on protein expression of TRAP, RANK, MMP-9 and Cts-K during osteoclastogenic differentiation

The protein expression of TRAP, RANK, MMP-9 and Cts-K of RAW264.7 cells on the 5th, 7th, and 9th day in different groups was shown in A, B and C of Fig. 3. The expression of the four osteoclast-specific factors in osteoclastogenic differentiation group was significantly higher than in other groups at each time point (P<0.05). In consistent with the regulatory effect of CORM-3 on the mRNA level of the osteoclast-specific genes, the protein expression TRAP, RANK, MMP-9 and Cts-K in CORM-3-osteoclastogenic differentiation group significantly decreased compared with the cells without CORM-3 pretreatment (P<0.05). With degassed CORM-3 pretreatment, the protein expression of the cells maintained the same level as that in osteoclastogenic differentiation group at each indicated time point (P>0.05), which suggested again that the regulatory effect of CORM-3 was mediated by CO releasing.

Selection of the lentiviral expression vector

The results of real-time fluorescence quantitative PCR showed that the expression of HO-1 gene in sh3 group was the least, compared with the blank group and the control group (P<0.05), as shown in A of Fig. 4A. Western blot results also showed that the HO-1 protein expression of sh3 group was lower than that in the blank group and the control group (P<0.05) (Fig. 4B). Based on the results of the data, sh3 lentivirus was selected for the following experiment.

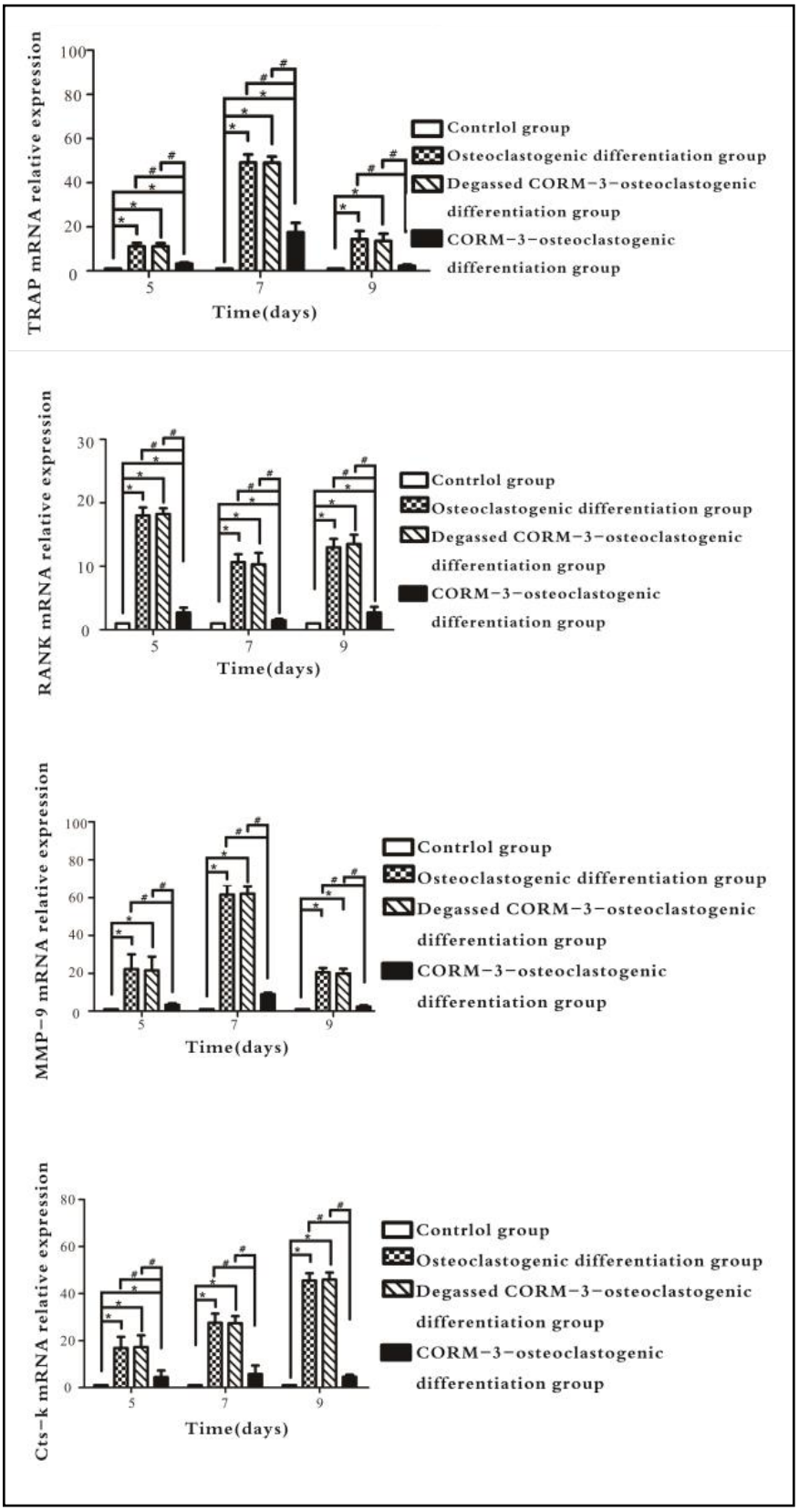

Fig. 2. Effect of CORM-3 on mRNA expression of TRAP, RANK, MMP-9 and Cts-K during osteoclastogenic differentiation. RAW264.7 cells were cultured in conditions indicated in different groups for 5, 7 and 9 days. The mRNA expression of TRAP, RANK, MMP-9 and Cts-K was assessed by RT-qPCR. Data were presented as the mean ± standard deviation (n=3). *: P<0.05 vs. control group; #: P<0.05 as indicated.

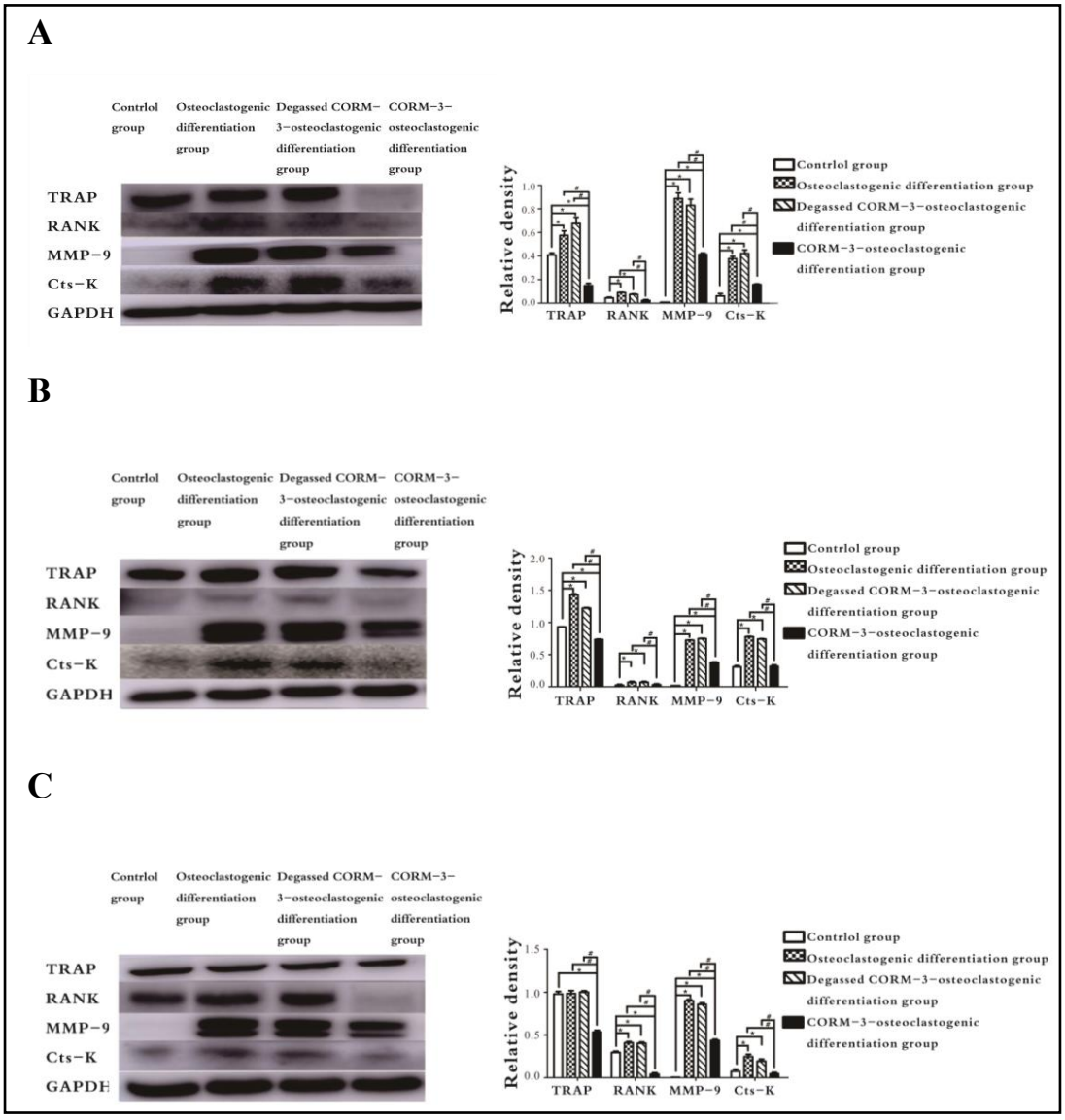

Fig. 3. Effect of CORM-3 on protein expression of TRAP, RANK, MMP-9 and Cts-K during osteoclastogenic differentiation. RAW264.7 cells were cultured in conditions indicated in different groups. The protein expression of TRAP, RANK, MMP-9 and Cts-K on day 5, 7 and 9 was indicated in A, B and C, respectively. Data were presented as the mean ± standard deviation (n=3). *: P<0.05 vs. control group; #: P<0.05 as indicated.

Effect of HO-1-shRNA transfection on mRNA expression of TRAP, RANK, MMP-9 and Cts-K

RAW264.7 cells were transfected with HO-1-shRNA lentivirus. Transfected cells were subjected to osteoclastogenic differentiation with or without CORM-3 pretreatment. On day 5, 7 and 9, the expression of TRAP, RANK, MMP-9 and Cts-K were detected by RT-qPCR. As shown in Fig. 5, the mRNA expression of TRAP, RANK, MMP-9 and Cts-K in osteoclastogenic differentiation group was significantly increased than control group on each time point (P<0.05). While the expression of these four osteoclast-specific genes in osteoclastogenic differentiation group was maintained the same level as that in CORM-3-osteoclastogenic differentiation group and degassed CORM-3-osteoclastogenic differentiation group (P>0.05). These results that CORM-3 was no more effective on the inhibition of the osteoclast-specific gene expression after HO-1-shRNA transfection suggested that the regulatory effect of CORM-3 was, at least partly, mediated by HO-1 pathway.

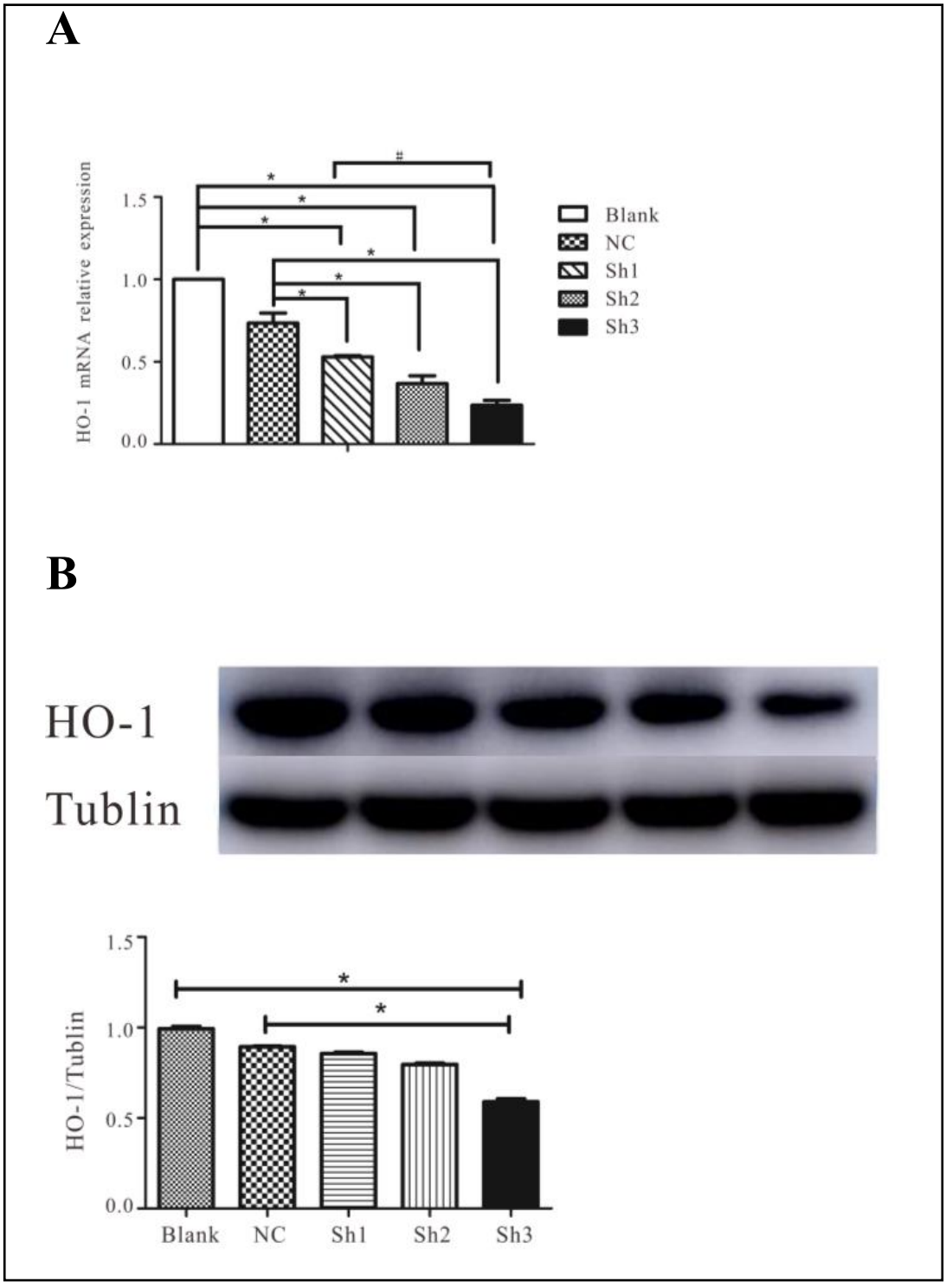

Fig 4. Selection of the lentiviral expression vector. The mRNA expression (A) and protein expression of HO-1 (B) in blank, NC, Sh1, Sh2 and Sh3 group was checked by RT-qPCR and western blot. *: P<0.05 vs. control.

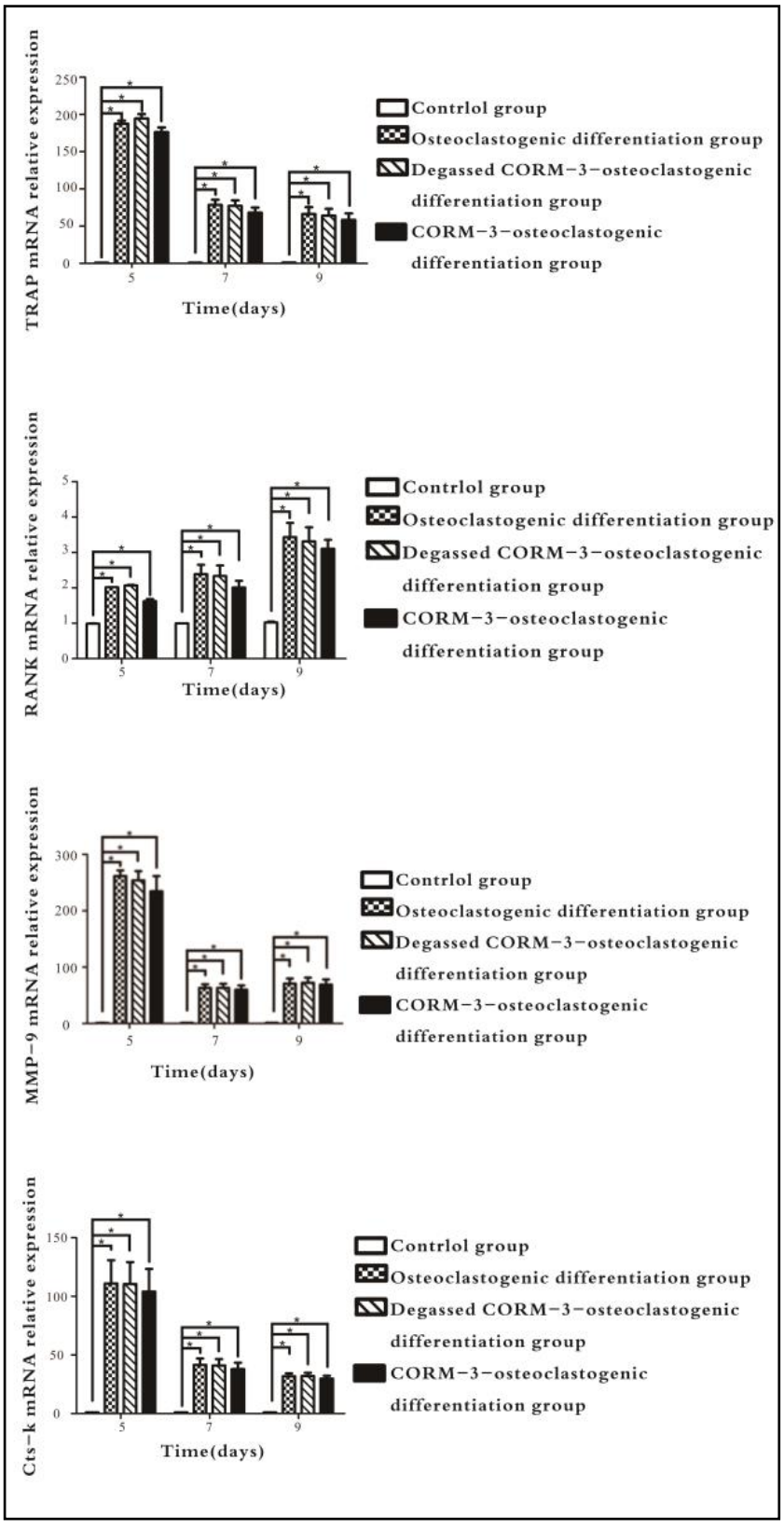

Fig. 5. Effect of HO-1-shRNA transfection on the mRNA expression of TRAP, RANK, MMP-9 and Cts-K. RAW264.7 cells were transfected with HO-1-shRNA lentivirus. Transfected cells were subjected to osteoclastogenic differentiation in conditions indicated in different groups for 5, 7 and 9 days. The mRNA expression of TRAP, RANK, MMP-9 and Cathepsin K was assessed by RT-qPCR. Data were presented as the mean ± standard deviation (n=3). *: P<0.05 vs. control group.

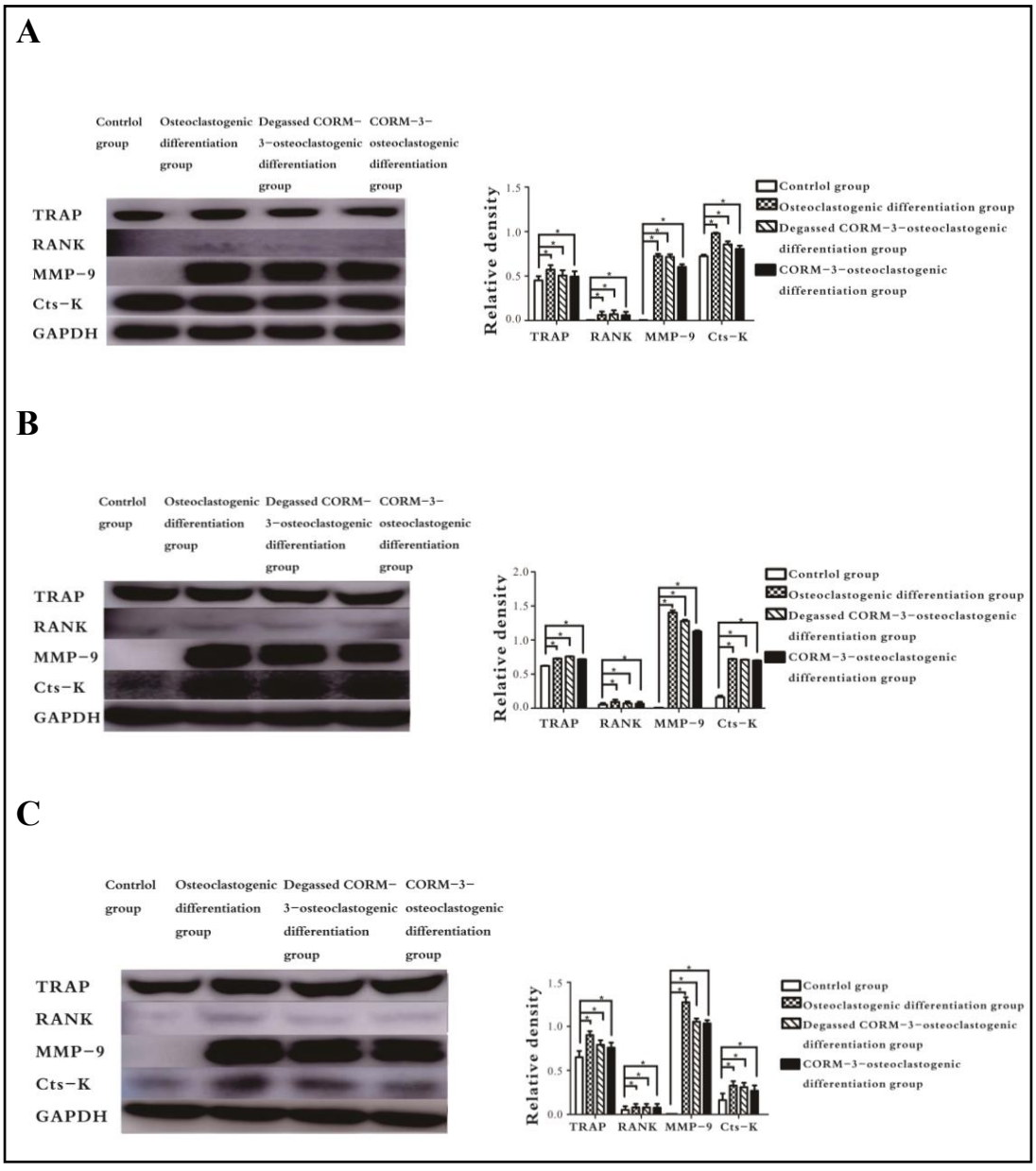

Fig. 6. Effect of HO-1-shRNA transfection on the mRNA expression of TRAP, RANK, MMP-9 and Cts-K. RAW264.7 cells were transfected with HO-1-shRNA lentivirus. Transfected cells were subjected to osteoclastogenic differentiation in conditions indicated in different groups. The protein expression of TRAP, RANK, MMP-9 and Cts-K was shown in A, B and C, respectively. Data were presented as the mean ± standard deviation (n=3). *: P<0.05 vs. control group.

Effect of HO-1-shRNA transfection on protein expression of TRAP, RANK, MMP-9 and Cathepsin K

The protein expression of TRAP, RANK, MMP-9 and Cts-K on day 5, 7, 9 was shown in Fig. 6A-C, respectively. The expression of these four osteoclast-specific factors in osteoclastogenic differentiation group was significantly higher than in control group (P<0.05), but maintained the same level as that in degassed CORM-3-osteoclastogenic differentiation group and CORM-3-osteoclastogenic differentiation group at different time point (P>0.05), which suggested that CORM-3 failed to regulate the expression after HO-1-shRNA transfection.

Discussion

Recent studies have demonstrated the protective effects of CO and CORMs *in vitro* and *in vivo* experiments. However, little is known regarding the influence of CORMs on osteoclastogenesis. In the present study, we used RAW264.7 cells as *in vitro* model to investigate the effects of CORM-3 on the osteoclastogenic differentiation of the cells, and explored the possible mechanism underlying the regulation of the CORM-3. The results of the study were as following: firstly, CORM-3 significantly promoted the proliferation of RAW264.7 cells at a concentration of 200 μM. Secondly, pretreatment with CORM-3 up-regulated the mRNA and protein expression of the osteoclast-specific markers TRAP, RANK, MMP-9 and Cathepsin K. Notably, the regulatory effect of CORM-3 was mediated by the release of CO. Thirdly, the effect of CORM-3 on the expression of TRAP, RANK, MMP-9 and Cathepsin K was partly mediated by HO-1 pathway.

The induction of RAW264.7 cells to obtain mature osteoclasts through RANKL is a widely used cell model in study on osteoclastogenesis. The differentiation of osteoclast, a process termed osteoclastogenesis, is a complex but orderly event, during which there are a series of landmark genes that can be used as markers for osteoclasts and their differentiation stages. Tartrate-resistant acid phosphatase (TRAP) is a key enzyme, which is highly expressed in osteoclasts and involved in osteoclast-mediated bone transformation. Cathepsin K (Cts-K) is expressed in osteoclasts and existed in the form of the collagenolytic cysteine protease. Both Cts-K and TRAP are important genes for the identification of osteoclasts [24]. Matrix metalloproteinase (MMP) belongs to the family of zinc-binding endopeptidase, which is required for the degradation of extracellular matrix of various tissues including bone tissue. MMP-9 is highly expressed in osteoclasts, which is an important protease that degrades extracellular matrix and participates in bone remodeling and plays an important role in osteoclast-activated bone resorption [25].

CORMs are a novel group of compounds that are carriers of CO and reproduce its biological actions [20]. There is an abundance of preclinical evidence in both large and small animal models of diseases demonstrating the protective effects of CO at low concentrations (15–250 ppm) and CORMs, including hyperacute endotoxic shock [26], pulmonary inflammation [27], postoperative ileus [28], organ transplantation-induced ischemia-reperfusion injury [29, 30-33], airway hyperresponsiveness [34], necrotizing enterocolitis [35], and pulmonary hypertension [36]. CORM-3 has also been reported to promote the osteogenic differentiation of bone marrow mesenchymal stem cells by releasing CO [37].

The heme oxygenase (HO) system is a microsomal enzyme system that widely exists in humans and mammals, which is the rate-limiting enzyme for heme degradation leading to generation of biliverdin, which is then further processed to bilirubin. To date, there have been reported three isoforms of HO, HO-1, HO-2 and HO-3. Of which, HO-1 is an inducible enzyme. It is expressed at low level under normal condition, but is up-regulated within short period in response to a host of cellular stressors including oxidants, pathogens, chemokine mediators, and growth factors [38]. Based on current evidence, the HO-1 pathway plays an important role in the control of inflammation. Overexpression of HO-1 modulates pro-inflammatory processes in a number of cells including macrophages and endothelial cells [39, 40]. Moreover, the anti-inflammatory role of HO-1 has been addressed in models dealing with human diseases, such as lupus erythematosus and rheumatoid arthritis [41-44], which are typically associated with bone loss. HO-1 is therefore regarded as a potential target for the treatment of inflammatory diseases.

There have been demonstrated that CORMs up-regulate the expression of HO-1 in *in vitro* and *in vivo* models [45-48]. In our preliminary experiment (data not shown), we found that both 200 and 400 umol/l CORM-3 up-regulated the expression of HO-1 significantly, while CORM-3 at 100umol/l did not increase the expression of HO-1 significantly. We therefore chose 200 umol/l, the less concentration which up-regulated the expression of HO-1, as the working concentration in the experiment.

Results of the present study showed that CORM-3 significantly increased the expression of HO-1 during the osteoclastogenic differentiation. However, CORM-3 lost its inhibitory effect on osteoclastogenic differentiation after HO-1-shRNA was transfected to the cells, which indicated that the effect of CORM-3 relied on HO-1.

Imbalanced bone homeostasis results in pathological inflammatory bone disease. Increased osteoclasts and excessive osteoclast activity lead to bone dysregulation and subsequently boss loss may account for the bone defect in many diseases, including inflammatory and degenerative diseases, and autoimmune diseases, such as chronic periodontitis, osteoporosis and rheumatoid arthritis [49, 50]. Osteoclast has therefore been regarded as a new target of therapeutic strategy for these pathological bone diseases. Studies have reported that CORM-3 can inhibit the expression of multiple inflammatory molecules in the lesion area of arthritic mice, and effectively improve its clinical characterization [51]. We also found that systemic administration of CORM-2 effectively suppresses the expression of TNF-α and IL-1β, and inhibits the alveolar bone absorption in rats with experimental periodontitis [52]. In the present study, we found that CORM-3 significantly suppressed the expression of osteoclast-specific markers TRAP, RANK, MMP-9 and Cathepsin K during osteoclastogenic differentiation of RAW264.7 cells, demonstrating the potential to inhibit oesteoclastogenesis. Degassed CORM-3 failed to inhibit the osteoclast-specific expression, suggesting the effect of CORM-3 is by CO.

CO mediates the toxic effects primarily by its strong affinity to hemoglobin and forming carboxyhemoglobin (COHb), thereby reducing the oxygen carrying capacity of the blood. The amount of CO inhaled and/or the exposure time are the most critical factors that determine the severity of CO poisoning. The level of COHb in healthy volunteers ranges from 1% to 6% proportion while in smokers may reach 14% which is generally considered tolerable [53]. The data published so far indicate that a 15–20% proportion of HbCO is, in the majority of cases, not detrimental and can be considered as the "biological threshold" for CO tolerance in humans [54]. The FDA has set COHb level 12–14% as the upper limit for human use based on Phase I human and animal studies [55]. In the preclinical experiments reported so far in the literature, the therapeutic doses of CO range from 15ppm to 500ppm, with the maximal COHb level reaching 12% [54, 56-58]. Inhaled CO is currently being developed in at least four clinical trials in North America in the context of paralytic ileus, pulmonary fibrosis, pulmonary hypertension, and organ transplantation (www.clinicaltrials.gov) [55]. In some researches in which CO-RMs were applied, pharmacologic effects were observed without altering the serum COHb levels [54, 59-61].

To date, only a few studies on therapeutic applications of CO inhalation in humans have been published. Bathoorn et al. reported a clinical study in which they demonstrated the feasibility of administering inhaled CO to humans with chronic obstructive pulmonary disease [62]. For future clinical use, CORMs can be designed in many ways which make them very attractive modes of delivery. For example, they can be created to deliver CO at the inflammatory site, where CO is released in response to elevated reactive oxygen species; designed to release CO in response to ultrasound waves or light; or carried in conjunction with tissueselective agents for diseasespecific targeting.

Conclusion

The results of the present study indicate that CORM-3 inhibits the osteoclastogenic differentiation of RAW264.7 cells via HO-1 pathway, which suggests CORM-3 may be developed as an available strategy for pathological bone defect disease. The influence of CORM-3 on bone defect disease needs to be investigated in more *in vivo* models. Although CORMs have potential to be used in treatment, further pharmacokinetic and toxicological analyses of CORMs are required prior to the clinical application.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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