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ORIGINAL ARTICLE

Pentamidine Inhibits Titanium Particle-Induced Osteolysis *In Vivo* and Receptor Activator of Nuclear Factor-κB Ligand-Mediated Osteoclast Differentiation *In Vitro*

Hye Jung Ihn[1] · Kiryeong Kim[2] · Hye-Sung Cho[2] · Eui Kyun Park[2]

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Abstract

BACKGROUND: Wear debris-induced osteolysis leads to periprosthetic loosening and subsequent prosthetic failure. Since excessive osteoclast formation is closely implicated in periprosthetic osteolysis, identification of agents to suppress osteoclast formation and/or function is crucial for the treatment and prevention of wear particle-induced bone destruction. In this study, we examined the potential effect of pentamidine treatment on titanium (Ti) particle-induced osteolysis, and receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis.

METHODS: The effect of pentamidine treatment on bone destruction was examined in Ti particle-induced osteolysis mouse model. Ti particles were implanted onto mouse calvaria, and vehicle or pentamidine was administered for 10 days. Then, calvarial bone tissue was analyzed using micro-computed tomography and histology. We performed *in vitro* osteoclastogenesis assay using bone marrow-derived macrophages (BMMs) to determine the effect of pentamidine on osteoclast formation. BMMs were treated with 20 ng/mL RANKL and 10 ng/mL macrophage colony-stimulating factor in the presence or absence of pentamidine. Osteoclast differentiation was determined by tartrate-resistant acid phosphatase staining, real-time polymerase chain reaction, and immunofluorescence staining.

RESULTS: Pentamidine administration decreased Ti particle-induced osteoclast formation significantly and prevented bone destruction compared to the Ti particle group *in vivo*. Pentamidine also suppressed RANKL-induced osteoclast differentiation and actin ring formation markedly, and inhibited the expression of nuclear factor of activated T cell c1 and osteoclast-specific genes *in vitro*. Additionally, pentamidine also attenuated RANKL-mediated phosphorylation of IκBα in BMMs.

CONCLUSION: These results indicate that pentamidine is effective in inhibiting osteoclast formation and significantly attenuates wear debris-induced bone loss in mice.

Keywords Pentamidine · Osteolysis · Osteoclastogenesis · Titanium · RANKL

1 Introduction

Total joint arthroplasty (TJA) is an effective treatment option to alleviate pain and restore physical function in patients suffering from serious joint diseases, such as osteoarthritis, and rheumatoid arthritis [1]. In spite of being a useful procedure, periprosthetic osteolysis caused by wear debris is considered to be a major complication, which subsequently results in aseptic loosening and failure of TJA [1]. Wear particles generated from prosthesis

✉ Eui Kyun Park
epark@knu.ac.kr

[1] Institute for Hard Tissue and Bio-tooth Regeneration (IHBR), Kyungpook National University, 2177 Dalgubeol-daero, Jung-gu, Daegu 41940, Republic of Korea

[2] Department of Oral Pathology and Regenerative Medicine, School of Dentistry, IHBR, Kyungpook National University, 2177 Dalgubeol-daero, Jung-gu, Daegu 41940, Republic of Korea

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generally triggers inflammatory reactions, whereby numerous cytokines and chemokines, such as tumor necrosis factor (TNF)-α, and interleukin (IL)-6 are released [2]. These mediators directly and indirectly promote expression of receptor activator of nuclear factor κB ligand (RANKL) in osteoblasts and activated T cells, which enhances the differentiation of precursors into mature osteoclasts and bone resorption [3, 4]. Given that excessive osteoclast formation and function have profound impact on wear debris-mediated osteolysis, suppression of osteoclastogenesis and/or bone resorption would be potential target to prevent periprosthetic osteolysis and subsequent aseptic loosening.

Two important cytokines, macrophage colony-stimulating factor (M-CSF) and RANKL, drive differentiation of monocyte/macrophage lineage precursor cells into multinucleated osteoclasts, which are responsible for bone remodeling and pathological osteolysis [5]. RANKL/RANK activates intracellular signaling pathways including mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to primarily support the differentiation and function of osteoclasts, which ultimately leads to the induction of nuclear factor of activated T-cell c1 (NFATc1) followed by upregulation of its target genes contributing to osteoclastogenesis [6]. Even though antiresorptive agents exert partial protective effect in animal models [7, 8], approved therapeutic treatments for osteolysis patients are currently unavailable [9, 10]. In addition, since the pathogenesis of wear particle-induced osteolysis involves both inflammation and bone resorption, a chemical compound that harbors both anti-inflammatory and antiresorptive effects may be effective for therapeutic intervention of wear debris-induced osteolysis.

Pentamidine isethionate is an antiprotozoal agent that is used to treat *Pneumocystis carinii* pneumonia (PCP), which is a potentially life-threatening disease in adults infected with human immunodeficiency virus (HIV), and it has also been shown to be effective against fungal and protozoan pathogens [11, 12]. Markowitz et al. [13] have revealed anticancer activity of pentamidine to prevent the formation of S100B-p53 complex in melanoma cancer cells. In addition to antiprotozoal and anticancer activity, *in vivo* experiments have also shown that pentamidine attenuates dextran sodium sulphate (DSS)-mediated intestinal inflammation [14]. Pentamidine also reduced the levels of tumor necrosis factor (TNF) and interleukin (IL)-6 from LPS injection [15, 16]. However, the pharmacological effects of pentamidine, an anti-inflammatory drug, on osteoclastogenesis and wear particle-mediated osteolysis have not been investigated. Therefore, we examined the effect of pentamidine on RANKL-induced osteoclast formation and bone resorption *in vitro,* and *in vivo* using titanium (Ti) particle-induced osteolysis in a mouse calvarial model.

2 Materials and methods

2.1 Ti particle-induced calvarial osteolysis model

Seven-week-old male C57BL/6 mice (Dae Han Bio Link, Eumseong, Chungbuk, Korea) were used to determine the effect of pentamidine on particle-induced calvarial osteolysis *in vivo*, as described previously [3]. Mice were divided into four groups (n = 5 per group): sham control (Control), Ti particle with vehicle (Ti), Ti particle with pentamidine 1 mg/kg/day (Ti + Pentamidine 1 mg/kg), and Ti particle with pentamidine 5 mg/kg/day (Ti + Pentamidine 5 mg/kg). The animals were anaesthetized and 15 mg of commercial pure Ti particles (99.9%, 30–50 nm, US Research Nanomaterials, Inc., Houston, TX, USA) in 30 μL phosphate buffered saline (PBS) were embedded under the periosteum at the center of the calvarium. Vehicle or pentamidine (1 or 5 mg/kg) was administered by intraperitoneal injection daily for 10 days. At the end of the treatment period, all mice were sacrificed, and the calvariae were isolated and fixed in 3.7% formaldehyde in PBS for micro-computed tomography (micro-CT) and histological analysis.

2.2 Micro-computed tomography (CT) and histomorphometric analysis

The calvariae were fixed and analyzed using a high-resolution micro-CT scanner (Skyscan 1272, Bruker, Kontich, Belgium) and scanned at a resolution of 12 μm with a voltage source of 60 kV and current of 166 μA. Three-dimensional images were reconstructed using the CTvox software, and a cylinder-shaped region of interest (ROI; 3 × 3 × 1 mm) around the midline suture was selected for further quantitative analysis, as described previously [17]. Bone mineral density (BMD) and bone volume to tissue volume (BV/TV) ratio were analyzed as described previously [18]. For histological analysis, calvariae were fixed, decalcified in 10% EDTA, and embedded in paraffin. Histological sections were stained with hematoxylin and eosin (H&E) or TRAP. The stained sections were examined under brightfield light microscopy (Leica Microsystems, Wetzlar, Germany), and the number of TRAP-positive osteoclasts per bone perimeter was quantitated.

2.3 Osteoclast differentiation

Osteoclast differentiation was performed as described previously [19, 20]. Briefly, bone marrow cells were

isolated from the hind leg bones of mice and incubated in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). After 24 h, floating cells were collected and cultured in α-MEM containing 10% FBS and 30 ng/mL of M-CSF to generate bone marrow derived macrophages (BMMs). To induce osteoclast differentiation, BMMs were cultured in osteoclastogenic medium (20 ng/mL RANKL and 10 ng/mL M-CSF) in the presence or absence of different doses of pentamidine isethionate (Selleck Chemicals, Houston, TX, USA). The medium was replaced every 2 days until osteoclast-like cells were formed. Cultured cells were then washed with PBS, fixed in 4% paraformaldehyde, and stained for TRAP activity by an Acid Phosphatase, Leukocyte (TRAP) staining kit (Sigma-Aldrich, St. Louis, MO, USA). Cells with more than 3 nuclei were scored.

2.4 Cell viability assay

The effect of pentamidine on the viability of BMMs was evaluated using methyl-thiazol-tetrazolium (MTT) assay as described previously [21]. BMMs were incubated in α-MEM supplemented with 10% FBS and 10 ng/mL of M-CSF in the presence or absence of different concentrations of pentamidine. MTT was added to each well and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm on a 96-well microplate reader (BioRad, Hercules, CA, USA) to calculate % cell viability.

2.5 Real-time PCR

BMMs were cultured in the presence or absence of 5 μM pentamidine in osteoclast-inducing medium (20 ng/mL RANKL and 10 ng/mL M-CSF) for 4 days. Total RNA was extracted using TRI-solution (Bioscience, Seoul, Korea) and 1 μg of RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was carried out by using a LightCycler 1.5 real-time PCR system (Roche Diagnostics, Basel, Switzerland) and SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). The sequences of mouse primer pairs used in this study are as follows: TRAP (*Acp5*), 5′-TCCCCAATGCCCCATTC-3′ and 5′-CGGTTC TGGCGATCTCTTTG-3′; Cathepsin K (*Ctsk*), 5′-GGCTG TGGAGGCGGCTAT-3′ and 5′-AGAGTCAATGCCTCC GTTCTG-3′; DC-STAMP (*Dcstamp*), 5′-CTTCCGTGGG CCAGAAGTT-3′ and 5′-AGGCCAGTGCTGACTAGGA TGA-3′; NFATc1 (*Nfatc1*), 5′-ACCACCTTTCCGCAA CCA-3′ and 5′-TTCCGTTTCCCGTTGCA-3′.

2.6 Western blot analysis

Cultured cells were lysed in RIPA buffer containing protease and phosphatase inhibitors, and bicinchoninic acid (BCA) assay was carried out to determine the concentration of proteins. Proteins (30 μg) were separated on 10% SDS-PAGE and the bands were transferred on to a nitrocellulose membrane (Whatman, Florham Park, NJ, USA). After blocking for 1 h, the membrane was incubated with specific primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase. Proteins were detected using a WesternBright ECL detection kit (Advansta, Menlo Park, CA, USA) on a chemiluminescence image analyzer (Azure Biosystems, Inc., Dublin, CA, USA).

2.7 Immunofluorescence staining

BMMs were seeded on coverslips and cultured in α-MEM supplemented with 20 ng/mL RANKL, and 10 ng/mL M-CSF in the presence or absence of 5 μM pentamidine. After 5 days of culture, cells were fixed and permeabilized with Triton X-100. The cells were blocked and incubated with NFATc1 antibody followed by incubation with Alexa Fluor 488-labeled secondary antibody. F-actin and nuclei were identified by using rhodamine-conjugated phalloidin (Cytoskeleton, Denver, CO, USA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Santa Cruz Biotechnology, Dallas, TX, USA), respectively. Cells were examined using a Leica DM 2500 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.8 Statistical analysis

The data are expressed as mean ± standard deviation (SD). All the experiments were repeated at least three times. Statistical analysis was performed using two-tailed Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test. $p < 0.05$ or $p < 0.01$ indicated a statistically significant difference between groups.

3 Results

3.1 Effect of pentamidine on Ti particle-induced osteolysis

To evaluate the effect of pentamidine on bone loss *in vivo*, a Ti particle-induced calvarial osteolysis model was used. As shown in Fig. 1A, micro-CT images showed that the bone erosion on the calvariae was observed in mice treated with Ti particle compared to the control group. However,

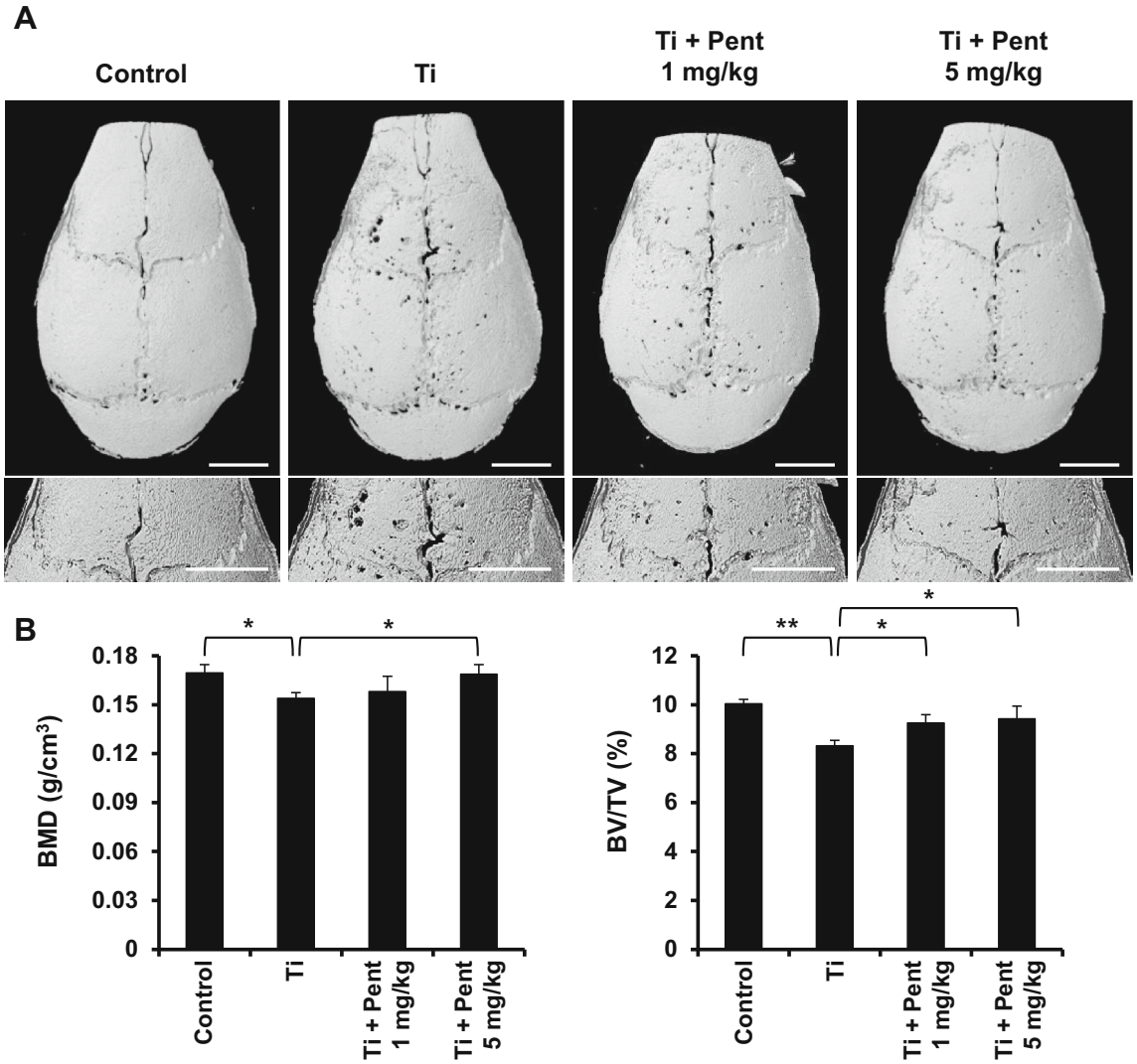

Fig. 1 Pentamidine prevented bone erosion in Ti particle-induced osteolysis in a mouse calvarial model. Ti particles were embedded on to the mouse calvaria, and injected with pentamidine (1 or 5 mg/kg) or vehicle control for 10 days. At the end of the study period, calvaria were dissected, fixed, and scanned with micro-CT. **A** Representative micro-CT images of calvaria from each group (n = 5). Scale bar = 5 mm. **B** Quantification of bone mineral density (BMD) and bone volume to tissue volume (BV/TV) ratio. $^{**}p < 0.01$ and $^{*}p < 0.05$. Pent: pentamidine

Ti particle-induced bone resorption decreased substantially in mice treated with pentamidine (Fig. 1A). Quantitative analysis of bone mineral density (BMD) and bone volume to tissue volume (BV/TV) ratio revealed significant reduction in Ti particle group, whereas pentamidine administration at a dose of 5 mg/kg daily led to a significant increase in these bone parameters (Fig. 1B).

Furthermore, histological analysis determined that pentamidine treatment attenuated bone erosion caused by Ti particle (Fig. 2A). Consistent with the micro-CT analysis of bone parameters, H&E staining revealed that treatment with pentamidine at a dose of 5 mg/kg daily protected against Ti-mediated bone loss (Fig. 2A). A significant increase in the number of TRAP-positive cells was observed along the eroded surface in the Ti particle treated group (Fig. 2A, arrowheads). However, treatment with pentamidine suppressed the formation of TRAP-positive cells (Fig. 2B).

3.2 Effect of pentamidine on RANKL-induced osteoclastogenesis

Further, we investigated whether pentamidine modulates RANKL-mediated osteoclast differentiation *in vitro*. BMMs were cultured in osteoclastogenic medium supplemented with vehicle or different concentrations of pentamidine. As expected, numerous TRAP-positive multinucleated cells were observed in cells treated with RANKL and M-CSF (Fig. 3A). However, treatment with pentamidine suppressed osteoclast differentiation

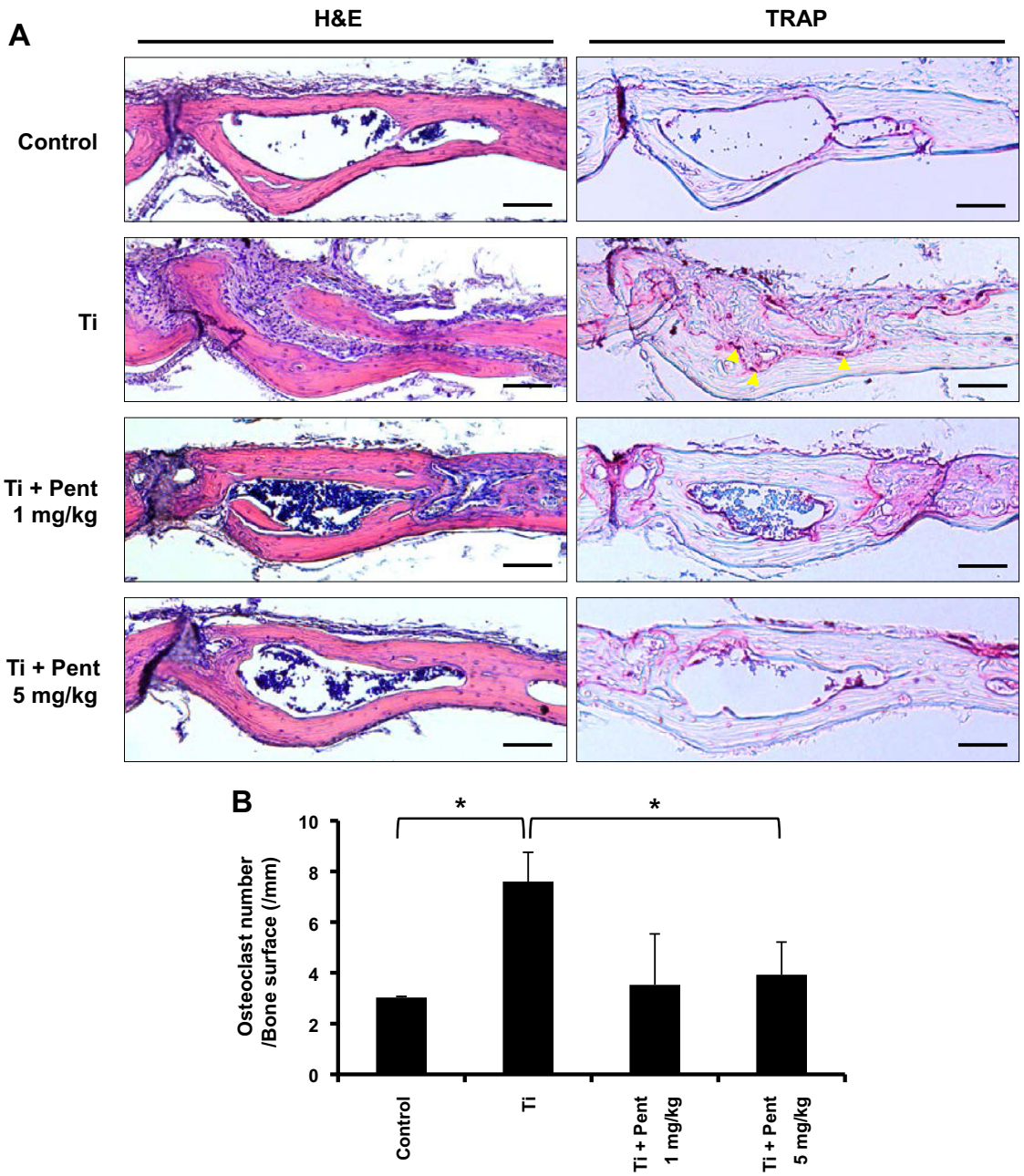

Fig. 2 Histological analysis of murine calvarial bone sections. **A** Representative images of hematoxylin and eosin (H&E), and tartrate-resistant acid phosphatase (TRAP) stained calvarial bone sections. Scale bar = 100 μm. **B** The number of TRAP-positive cells (yellow arrow head) per bone surface in each group (n = 5) was assessed (lower graph). *$p < 0.05$ versus Ti group. Pent: pentamidine

considerably, and the number of osteoclast-like cells were reduced by more than 90% in BMMs treated with 5 μM pentamidine (Fig. 3A, B). To determine whether anti-osteoclastogenic activity of pentamidine was due to its cytotoxic effect, we performed MTT assay. Pentamidine was not found to be cytotoxic to BMMs at concentrations below 10 μM (Fig. 3C). These results indicated that pentamidine suppressed RANKL-mediated osteoclast differentiation without exerting any cytotoxic effect on BMMs.

3.3 Effect of pentamidine on the expression of osteoclast-specific genes and the formation of actin rings

To further confirm the inhibitory effect of pentamidine on osteoclastogenesis, mRNA expression of *Nfatc1*, and its target genes, such as *Acp5*, *Ctsk*, and *Dcstamp* was assessed. Treatment with RANKL led to a significant increase in the mRNA levels of *Nfatc1*, however, treatment with pentamidine not only inhibited RANKL-mediated increase

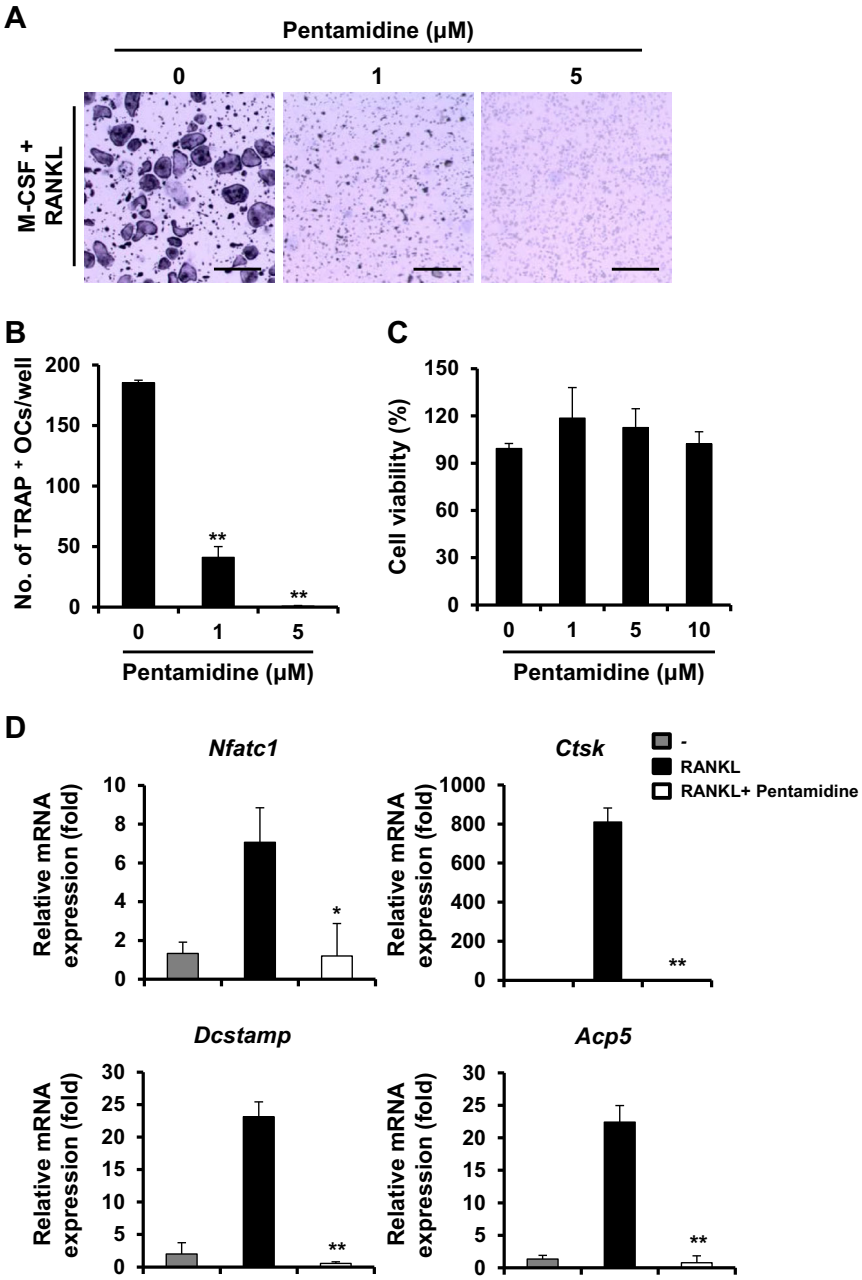

Fig. 3 Pentamidine suppressed RANKL-induced osteoclast differentiation *in vitro* and the expression of osteoclast-specific genes. **A** Bone marrow-derived macrophages (BMMs) were cultured in osteoclast-inducing medium containing M-CSF (10 ng/mL) and RANKL (20 ng/mL) in the presence or absence of various concentrations of pentamidine. After 4 days of culture, cells were fixed and stained for TRAP. Scale bar = 500 μm. **B** The number of TRAP-positive multinucleated cells was counted. **C** BMMs were treated with different doses of pentamidine in the presence of M-CSF for 3 days. Cell viability was determined by using MTT assay. **D** BMMs were incubated with M-CSF (10 ng/mL) and RANKL (20 ng/mL) in the presence or absence of 5 μM pentamidine for 4 days and mRNA levels of NFATc1 (*Nfatc1*), Cathepsin K (*Ctsk*), DC-STAMP (*Dcstamp*), and TRAP (*Acp5*) were determined by real-time PCR. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus vehicle-treated control group

in the mRNA levels of *Nfatc1* significantly but also its nuclear expression (Figs. 3D, 4A). Consistent with the reduced expression of *Nfatc1* in pentamidine-treated cells, mRNA levels of its target genes were also found to be significantly downregulated (Fig. 3D). DC-STAMP is required for the fusion of osteoclast precursors to form multinucleated osteoclasts [22]. In accordance with the decreased mRNA levels of *Dcstamp*, cells treated with pentamidine rarely made osteoclast-like giant cells as well as peripheral actin rings (Fig. 4A).

3.4 Effect of pentamidine on RANKL-dependent signaling pathways

To investigate the molecular mechanism by which pentamidine inhibits osteoclastogenesis, BMMs were pretreated with vehicle or 5 μM pentamidine followed by stimulation with RANKL. We examined whether treatment with pentamidine affected RANKL-dependent activation of MAPKs and NF-κB. As shown in Fig. 4B, pretreatment with pentamidine attenuated RANKL-mediated IκBα phosphorylation, although activation of MAPKs, such as p38, ERK, and JNK did not get affected, indicating that

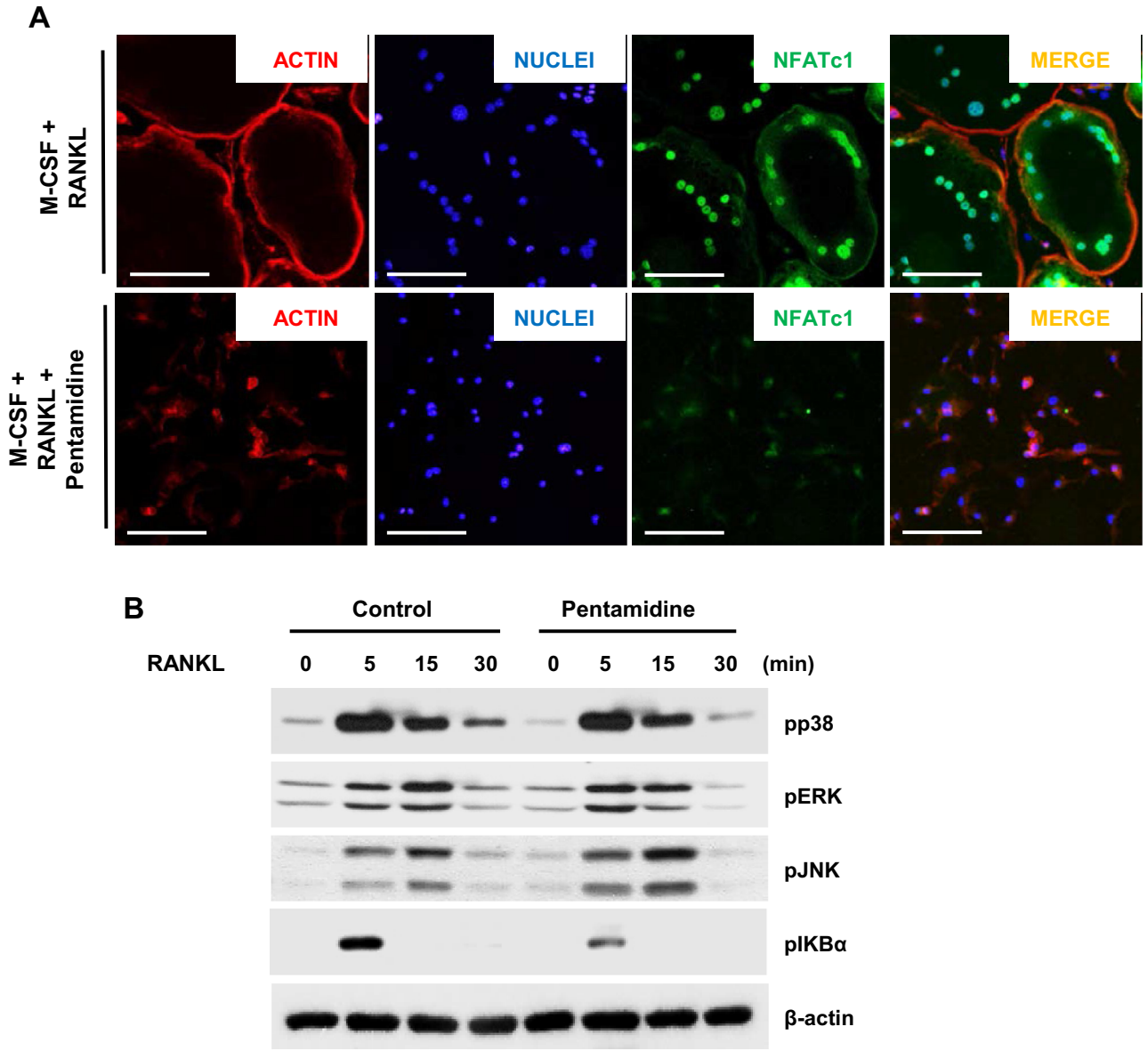

Fig. 4 Pentamidine inhibited actin ring formation and nuclear localization of NFATc1, and impaired RANKL-induced NF-κB signaling. **A** BMMs were seeded on glass coverslips and stimulated with M-CSF (10 ng/mL) and RANKL (20 ng/mL) in the presence or absence of 5 μM pentamidine. Nuclear localization of NFATc1 was examined by immunostaining. Nuclei and F-actin were labeled with DAPI and rhodamine-conjugated phalloidin, respectively. Scale bar = 50 μm. **B** Serum-starved BMMs were pretreated with or without 5 μM pentamidine for 1 h followed by stimulation with RANKL (50 ng/mL). Western blot analysis was performed to determine the levels of phosphorylated p38 (p-p38), p-ERK, p-JNK, and p-IκBα by using specific antibodies

pentamidine exerted anti-osteoclastogenic activity via modulating the NF-κB signaling pathway.

4 Discussion

It is considered that wear particle mediated inflammatory reaction and osteoclastic bone resorption around the implants are the principal pathogenic mechanisms responsible for periprosthetic osteolysis [2, 23]. In spite of the improvement in the quality of the implant materials, wear particles can be generated and they are capable of stimulating inflammatory responses and subsequent osteoclast formation [24]. Thus, osteoclasts and inflammation might serve as critical targets for therapeutic intervention to ameliorate and prevent wear debris-mediated bone loss after TJA.

Pentamidine has been known to exhibit various pharmacological properties, such as antiprotozoal, anticancer, and anti-inflammatory activities [12, 13, 16]. Given the

inflammation plays an important role in osteoclast differentiation, we hypothesized that treatment with pentamidine could prevent wear particle-induced bone loss caused by excessive osteoclast formation and function. Therefore, we aimed to determine the potential effect of pentamidine on osteoclast differentiation *in vitro,* and *in vivo* by using Ti particle-induced bone destruction in a murine calvarial model. In the present study, both micro-CT and histological analysis revealed that Ti particles stimulated bone destruction compared to the sham group, whereas pentamidine ameliorated Ti particle-induced calvarial bone resorption in mice (Figs. 1, 2). The number of osteoclasts responsible for Ti particle-induced bone resorption reduced significantly in Ti + Pentamidine (5 mg/kg) group compared to the Ti particle group, suggesting that pentamidine suppressed Ti particle mediated stimulation of osteoclast formation, and exhibited therapeutic potential against wear particle-mediated bone destruction.

Precursor cells of monocyte/macrophage lineages undergo several steps, such as proliferation, cellular fusion, and activation during osteoclast differentiation [25]. RANKL/RANK signaling required for osteoclast differentiation and bone resorbing function increased the expression of NFATc1, a major transcription factor, which in turn leads to the increased expression of osteoclast marker genes by activating several downstream signaling events [6]. Among the signaling cascades, the critical role of NF-κB signaling in osteoclast development has been established by demonstrating that NF-κB double-knockout mice developed osteoporosis as a result of a defect in osteoclast differentiation [26, 27]. In addition to this, NF-κB signaling is also known to be essential for the pathogenesis of osteolytic diseases including periprosthetic osteolysis and periodontitis [28]. The present study demonstrated that treatment with pentamidine prevented phosphorylation of IκBα, however, it did not affect RANKL-induced activation of MAPKs (Fig. 4B). Thus, these results indicate that pentamidine exhibits anti-osteoclastogenic activity by inhibiting NF-κB signaling pathway.

Ectopic overexpression of NFATc1 promotes differentiation of precursor cells into osteoclasts even in the absence of RANKL stimulation, and embryonic stem cells from mice deficient in NFATc1 were unable to generate osteoclasts in response to RANKL stimulation, demonstrating that NFATc1 is not only essential but also sufficient for osteoclast differentiation [29]. In the present study, treatment with pentamidine downregulated the expression of *Nfatc1* significantly at mRNA level, which subsequently resulted in inhibition of expression of its target genes, such as *Acp5*, *Ctsk*, and *Dcstamp* (Fig. 3D). In particular, DC-STAMP is a key regulator of cell–cell fusion which results in generation of multinucleated giant osteoclasts, and rearrangement of cytoskeletal features resulting in the formation of actin rings and ruffled borders [22] which are required for proper functioning of osteoclasts. Consistent with the decreased expression of *Dcstamp*, treatment with pentamidine attenuated multinucleation and the formation of actin rings (Fig. 4A). Collectively, our data reveal that pentamidine suppressed RANKL-mediated osteoclastogenesis by downregulating the expression of *Nfatc1*.

In summary, pentamidine suppressed osteoclast differentiation and the expression of osteoclast-specific marker genes by preventing the activation of NF-κB signaling pathway and attenuating RANKL-mediated induction of NFATc1 *in vitro*. Furthermore, pentamidine protected against wear debris-induced bone destruction in the murine calvarial osteolysis model. Thus, these findings suggest that pentamidine could have therapeutic potential for the treatment of osteolytic bone diseases that are associated with inflammation and excessive osteoclast formation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All animal experiments were approved by the committee on the care and use of animals in research at Kyungpook National University, South Korea, and conducted in accordance with the guidelines for the Care and Use of Laboratory Animals (2015-150).

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