

Cathepsin K Inhibitor Regulates Inflammation and Bone Destruction in Experimentally Induced Rat Periapical Lesions

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Abstract

Introduction: Cathepsin K is highly expressed in osteoclasts and plays an essential role in bone resorption. NC-2300 is an artificially designed cathepsin K inhibitor, and its application to experimentally induced arthritis induces down-regulation of bone destruction. In this study, we evaluated the effects of NC-2300 on inflammation and bone destruction in experimentally induced rat periapical lesions. **Methods:** The dental pulps of lower first molars in rats were extirpated, and the pulp chambers were left open to the oral environment. NC-2300 and phosphate-buffered saline were administered orally twice a day in the experimental and control groups, respectively. Animals were sacrificed on day 21, and the mandibles were extracted. The left hemimandibles were used for micro-computed tomographic and histologic examination. For the right hemimandibles, RNA was extracted from the periapical tissues surrounding the root apices, and inflammatory mediator expression was examined by real-time polymerase chain reaction using complementary DNA converted from extracted RNA. **Results:** The size of the periapical lesion, number of tartrate-resistant acid phosphatase-positive osteoclasts and major histocompatibility complex class II molecule-expressing macrophages in the experimental group decreased significantly when compared with the control group. The expression of proinflammatory cytokines in the experimental group was significantly suppressed when compared with the control group. **Conclusions:** These results suggest that the cathepsin K inhibitor may inhibit not only cathepsin K activity in osteoclasts but also inflammatory mediator synthesis relating to osteoclastogenesis, and these synergistic effects may be involved in the suppression of periapical lesion expansion. (*J Endod* 2015;41:1474–1479)

Key Words

Cathepsin K inhibitor, major histocompatibility complex class II molecules, osteoclasts, periapical bone destruction, periapical lesions, proinflammatory cytokines

Periapical lesions, including periapical granulomas, contain a variety of inflammatory cells that infiltrate the periapical tissues in response to bacteria and their toxins found in infected root canals. These immune responses are similar to those elicited by bacterial infections elsewhere in the body, but the typical feature of periapical lesions is the destruction of alveolar bone surrounding the root apex. We previously reported that macrophages are recognized as one of the most prevalent inflammatory cells in the periapical lesions (1–3). Furthermore, several cytokines that are mainly produced by macrophages, including interleukin (IL)-1, tumor necrosis factor, and IL-6, activate osteoclast formation and induce periapical bone destruction (4, 5). The synthesis of these bone-resorptive cytokines is regulated by a network of immunoregulatory cytokines, which are mainly produced by T cells. Generally, cytokines derived from type 1 helper (Th1) T cells (eg, interferon gamma, IL-2, and IL-12) promote inflammation and bone-resorptive cytokine expression, whereas type 2 helper (Th2) T-cell-derived cytokines (eg, IL-4 and IL-10) down-regulate the production of bone-resorptive cytokines and Th1-type cytokines. It is reported that IL-10 is an important endogenous suppressor of infection-stimulated bone resorption, likely acting via the inhibition of IL-1 α expression (6). The balance between Th1 and Th2 T-cell-derived cytokines determines the progression of inflammation and periapical bone resorption. Continuous bacterial stimuli from infected root canals induce a predominant shift to Th1 responses, which induces further bone destruction and lesion expansion (4, 7, 8). To control the inflammatory bone destruction in periapical lesions, it is essential to regulate the synthesis of these inflammatory mediators. The control of inflammation and/or bone resorption associated with periapical lesions has previously been reported in a number of studies (9–11).

Periapical bone destruction in periapical lesions is performed by osteoclasts, and down-regulation of osteoclast activity or osteoclastogenesis should enable the control of lesion expansion directly. Cathepsin K is a type of cysteine protease and is highly expressed in osteoclasts. Its major function is the degradation of a series of proteins, including type I collagen, which subsequently induces tissue destruction (12). In other words, cathepsin K is responsible for the bone resorption caused by osteoclasts. For the regulation of osteoclast activity, several cathepsin K inhibitors have been developed. NC-2300 (Nippon Chemiphar Co Ltd, Saitama, Japan) is an artificially designed cathepsin K inhibitor that binds to the active center of cathepsin K and blocks enzymatic action of cathepsin K strongly and specifically. NC-2300 predictably suppressed bone destruction in an experimentally induced arthritis model in mice by the inhibition of cathepsin K in osteoclasts. Interestingly, it also suppressed joint inflammation. This anti-inflammatory effect of NC-2300 was presumably caused by the inhibition of signaling by Toll-like receptor 9 in dendritic cells (13). Rheumatoid arthritis is a typical inflammatory lesion resulting in bone destruction and is therefore similar to periapical lesions. As such, NC-2300

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may suppress inflammation and bone destruction even in the periapical lesion. In this study, we evaluated the effects of NC-2300 on inflammation and bone destruction in experimentally induced rat periapical lesions.

Materials and Methods

Induction of Periapical Lesion and Drug Administration

Six-week-old Wistar rats ($n = 12$; Clea Japan, Tokyo, Japan) were anesthetized with ketamine hydrochloride and xylazine via intraperitoneal injection (90 mg/kg and 10 mg/kg, respectively), and pulp exposures were made through the occlusal surfaces of their lower first molars with no. 1/2 round burs as previously reported (1–3). Dental pulps were extirpated with no. 20 K-files and left open to the oral environment. The unexposed molars of 6-week-old rats ($n = 3$) were used as negative controls. At 21 days after cavity preparation, the animals were sacrificed by decapitation under ether anesthesia. All animal protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and experiments were performed under the control of the university's guidelines for animal experimentation (No. 0120156A).

NC-2300 (150 mg/kg) and saline were administered orally twice a day throughout the experimental period (days 0–21) to the experimental and positive control groups, respectively.

Micro-computed Tomographic Analysis

The left lower jaw was dissected and fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C for 8 hours for micro-computed tomographic (micro-CT) analysis using cone-beam tomography (SMX-90CT; Shimadzu Corp, Kyoto, Japan). The jaw was placed on a holder in the prone position, and the median plane was fitted to the z-axis of a computed tomographic scanning system. Microtomographic slices were acquired from each sample at 200- μ mol/L increments covering the entire mediolateral width of the mandible, and a series of coronal computed tomographic images were recorded. The periapical radiolucency surrounding the distal root on the representative sections, which included the mesial and distal roots of the mandibular first molar and those exhibiting a patent distal root canal apex, was determined with the use of a standard template superimposed on the periapical region of the distal root. The lesion was then qualified using Adobe Photoshop (Adobe Systems Inc, San Jose, CA), and the results were expressed in mm² of the mean $\pm$ standard deviation.

Histologic Examination

After micro-CT analysis, the samples were decalcified in 14% EDTA at 4°C for 3 to 4 weeks, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetech Co Ltd, Tokyo, Japan), and rapidly frozen in dry ice/hexane. Serial sections (7- μ m thick) were cut with a cryostat. General histologic evaluation was performed on hematoxylin-eosin-stained sections, and osteoclasts and preosteoclasts were determined on tartrate-resistant acid phosphatase (TRAP) using 1.3 mmol/L naphthol AS-BI phosphate (Sigma-Aldrich, St Louis, MO), 1.6 mmol/L Fast Red Violet LB salt (Sigma-Aldrich), and 50 mmol/L sodium tartrate in 100 mmol/L acetic acid buffer (pH = 5.2). The number of TRAP-positive osteoclasts in the periapical lesion of the distal root of lower first molars was counted, and their density on the alveolar bone surface was calculated by their number per millimeter of bone surface in the periapical lesions.

Immunohistochemical staining using anti-major histocompatibility complex (MHC) class II antigen monoclonal antibody (MCA46G; OX6; 1:5,000 dilution; Serotec, Kidlington, UK) was performed using the avidin-biotin-peroxidase complex method. The antibody was diluted in 0.05 mol/L Tris(hydroxymethyl)aminome-

thane hydrochloride (Tris-HCl) buffer (pH = 7.6) containing 154 mmol/L NaCl (Tris-buffered saline [TBS]). After blocking endogenous peroxidase activity with 3% H₂O₂ for 10 minutes, the sections were incubated with the primary antibody overnight at 4°C. The sections were subsequently incubated with biotinylated horse antimouse immunoglobulin G (1:1000 dilution; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature followed by a further incubation with avidin-biotin-peroxidase complex reagent (Elite ABC Kit, Vector Laboratories) for 30 minutes. Each step was followed by 3 washes with TBS for 5 minutes each. Color was developed with 3,3'-diaminobenzidine HCl, and the sections were counterstained with methyl green and examined under a light microscope. Negative control staining was performed by replacing the primary antibody with TBS or normal mouse immunoglobulin G (Vector Laboratories). The number of OX6-positive cells in the periapical area of the distal root was counted in 10 randomly selected high-power fields using a 10 $\times$ 10 mm ocular grid. Results were expressed as the mean count of cells per high-power field.

Quantitative Real-time Reverse-transcription Polymerase Chain Reaction

The periapical tissue of the first molar with the root apex was dissected from the right lower jaw of each rat under a microscope. The total RNA of each sample was isolated from the periapical lesions and surrounding alveolar bone tissues with an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 300 ng total RNA using Moloney Murine Leukemia Virus RNase H reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA) and subjected to real-time polymerase chain reaction (qPCR) using Taq polymerase and SYBR green (Platinum SYBR Green qPCR SuperMix-UDG, Invitrogen) in a qPCR thermal cycler (DNA Engine Opticon; Bio-Rad, Hercules, CA). The PCR primers used were designed using the Primer 3 software (<http://primer3.sourceforge.net>). Specific primers and product sizes (in parentheses) were as follows:

1. Rat beta-actin: 5'-tgaccacactgggacgata-3' and 5'-cttttcacgggtggccttag-3' (130 bp)
2. IL-1 α : 5'-caccttctgctctaaagtgc-3' and 5'-aattctgctgctgaggatgc-3' (295 bp)
3. IL-6: 5'-ccggagaggagacttcacag-3' and 5'-cagaattgccattgcacaac-3' (134 bp)

The temperatures and times for denaturation, annealing, and elongation were 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, respectively, which were repeated for 40 cycles. The expression level of each gene was normalized to the corresponding beta-actin expression level.

Nitric Oxide Production by Lipopolysaccharide-stimulated RAW264 Cells *In Vitro*

The mouse macrophage cell line RAW264 (obtained from Riken BRC, Tsukuba, Japan) was cultured with the presence or absence of NC-2300 (10 μ mol/L) for 24 hours in Dulbecco-modified Eagle medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT). Lipopolysaccharide (LPS) (100 ng/mL, *Escherichia coli* O55:B5, Sigma-Aldrich) was then applied, and the cells were further cultured for 20 hours. Non-LPS-stimulated RAW264 were used as controls. The production of nitric oxide was examined using Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylene-diamine dihydrochloride, and 2.5% phosphoric acid; Sigma-Aldrich).

Statistical Analysis

The results were analyzed by 1-way analysis of variance with the Tukey-Kramer post hoc test.

Results

Periapical Bone Loss Examined by Micro-CT Imaging

Unexposed molars exhibited no periapical bone resorption (Fig. 1C), whereas infected animals showed increased bone resorption (Fig. 1A and B). The size of the periapical bone resorption in the NC-

2300 group was significantly smaller when compared with that in the control group ($P < .05$, Fig. 1D).

Histologic Examination

Hematoxylin-eosin staining showed abscess formation around the root apex and various neutrophils, and macrophages were present in the periapical lesions on day 21 in both groups. The size of the periapical lesions in the NC-2300 group was smaller than those in the control group (Fig. 1E and F). Unexposed molars showed no periapical lesion formation in either the control or the NC-2300 group.

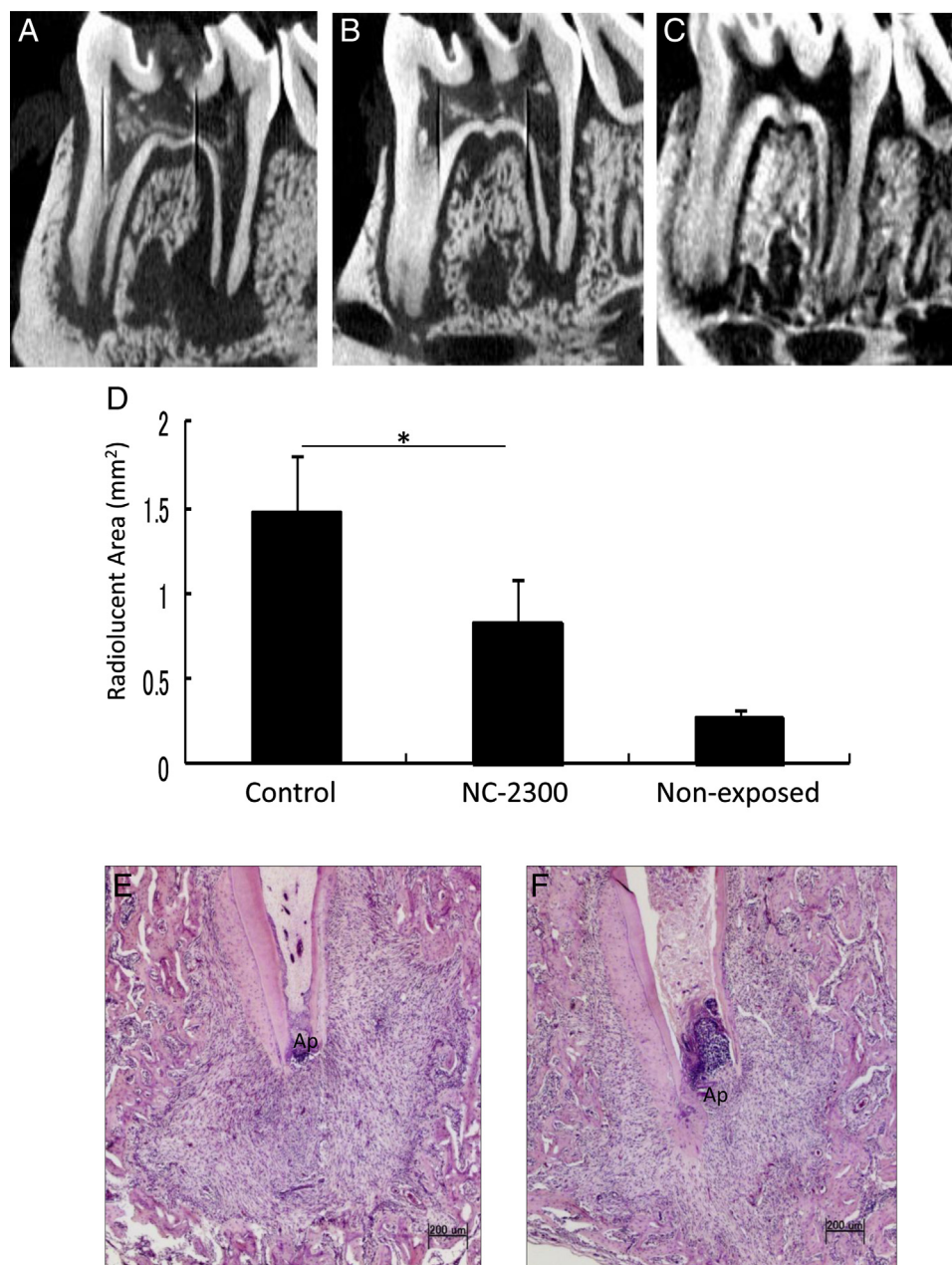


Figure 1. Micro-CT imaging and histologic evaluation of the rat periapical lesions. Micro-CT imaging revealed obvious periapical bone destruction in the (A) control and (B) NC-2300 group. (C) No periapical bone resorption was exhibited in the unexposed molars. (D) The size of periapical bone resorption in the NC-2300 group was significantly smaller when compared with the control group. $*P < .05$. Abscess formation around the apex (Ap) and establishment of periapical lesions were observed on hematoxylin-eosin-stained sections in the (E) control and (F) NC-2300 group. The sizes of the periapical lesions in the NC-2300 group were smaller than in the control group (Scale bars = 200 µm).

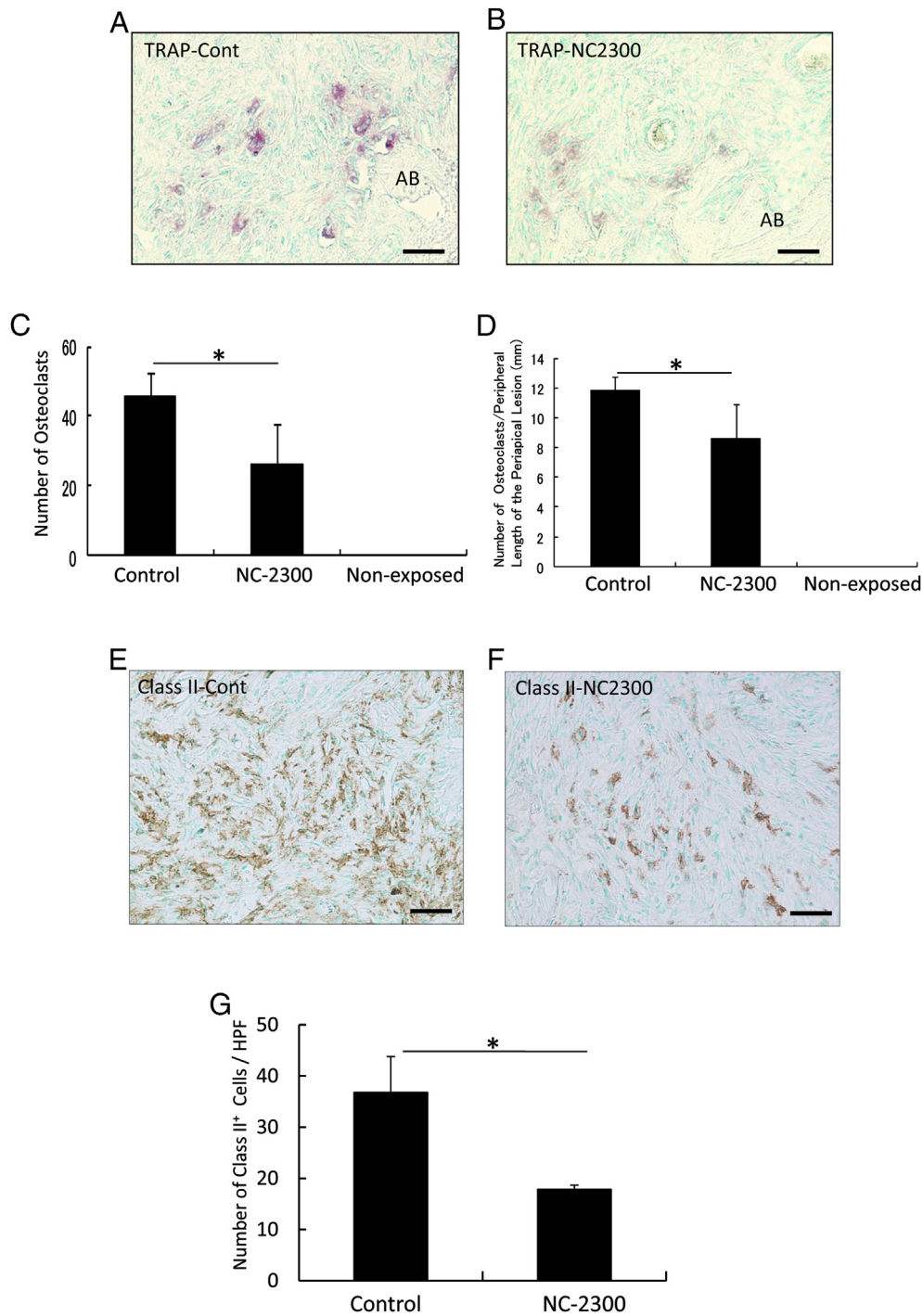


Figure 2. TRAP-positive osteoclasts and MHC class II molecule-expressing macrophages in rat periapical lesions. TRAP-positive osteoclasts were found on the surface of the alveolar bone (AB) in the (A) control and (B) NC-2300 group (Scale bars = 50 μ m). The (C) number and (D) density of TRAP-positive osteoclasts in the NC-2300 group were significantly smaller than in the control group. $*P < .05$. The infiltration of MHC class II molecule expressing macrophages was observed in the periapical lesions of the (E) control and (F) NC-2300 group (Scale bars = 50 μ m). (G) The density of MHC class II molecule expressing macrophages in the NC-2300 group was significantly lower than in the control group. $*P < .05$. HPF, high-power field.

TRAP-positive osteoclasts were observed on the bone surface in both groups. The number and density of osteoclasts in the NC-2300 group were significantly smaller than those in the control group ($P < .05$, Fig. 2A–D).

The infiltration of various MHC class II molecule-expressing macrophages were observed in the periapical lesions of both groups. These macrophages showed various morphologies including spindle-like, dendritic, and round. The density of these activated macrophages in the

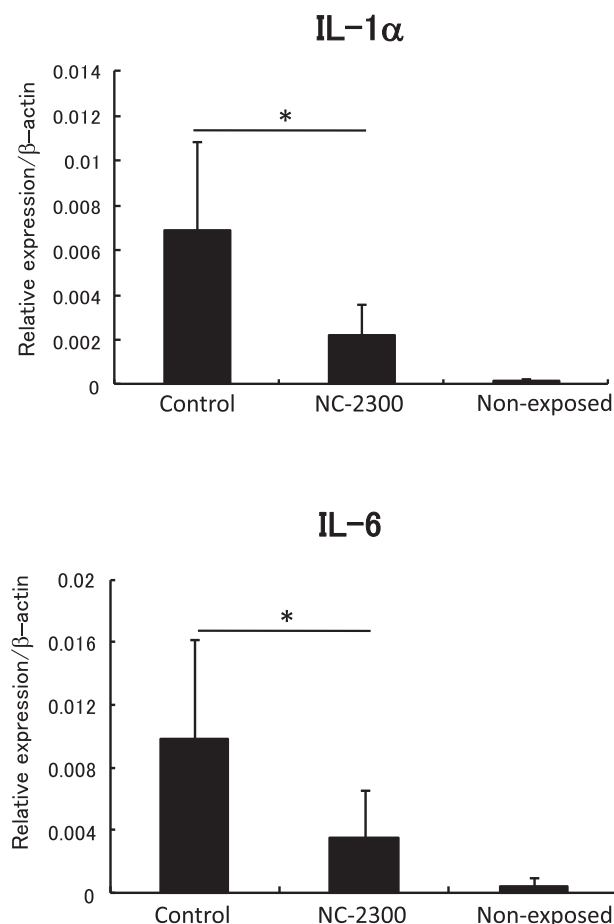


Figure 3. Messenger RNA expression of proinflammatory cytokines in rat periapical lesions. Messenger RNA expression of proinflammatory cytokines in the NC-2300 group was significantly suppressed when compared with the control group. $*P < .05$.

NC-2300 group was significantly lower than that in the control group ($P < .05$, Fig. 2E–G).

Proinflammatory Cytokine Expression in the Periapical Lesion

The expression of proinflammatory cytokines (IL-1 α and IL-6) in the periapical lesions of the NC-2300 group was significantly

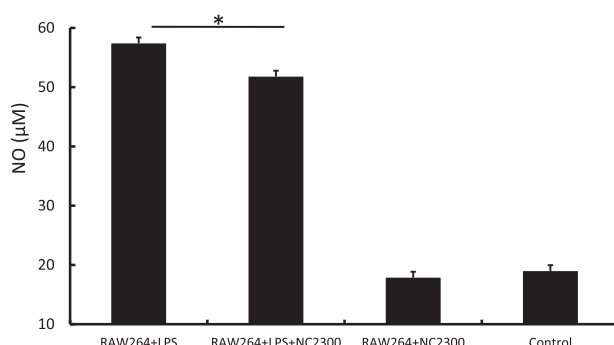


Figure 4. Nitric oxide synthesis from LPS-stimulated RAW264 macrophages. Nitric oxide (NO) synthesis from RAW264 macrophages in response to LPS was significantly suppressed by pretreatment with NC-2300. $*P < .05$.

suppressed when compared with that of the control group ($P < .05$, Fig. 3).

Nitric Oxide Production by LPS-stimulated RAW264 Cells

Nitric oxide synthesis from LPS-stimulated RAW264 macrophages increased significantly compared with that from nonstimulated macrophages, whereas pretreatment of NC-2300 significantly prohibited nitric oxide synthesis from LPS-stimulated macrophages. NC-2300 had no effect on nitric oxide synthesis in the absence of LPS ($P < .05$, Fig. 4).

Discussion

Our study showed that the size of the periapical lesion in the NC-2300 group was significantly smaller when compared with the control group as assessed by micro-CT analysis. This suggests that NC-2300 effectively suppressed the expansion of periapical lesions. Lesion expansion accompanied by periapical bone destruction is carried out by osteoclasts. A decrease in the number of osteoclasts in the periapical lesion should be responsible for the down-regulation of lesion expansion in the NC-2300 group. The induction of osteoclasts is mediated by proinflammatory mediators, such as IL-1 and IL-6, which are macrophage-derived proinflammatory cytokines (14, 15). These are deeply involved in bone resorption in periapical lesions, especially IL-1 α , which is strongly implicated as the primary stimulator of periapical bone destruction (16). IL-1 α increases receptor activator of NF- κ B ligand (RANKL) expression, which is the direct inducer of osteoclasts (17). IL-6 is one of the essential mediators for the induction of T helper 17 cells (Th17) (18), and Th17 synthesizes IL-17, which is also a prominent inducer of RANKL (19). We revealed that messenger RNA expression of IL-1 α and IL-6 was up-regulated in the exposed group when compared with the nonexposed group, which is coincident with previous reports (4). In contrast, the expression of these proinflammatory mediators increased even in the NC-2300 group but was significantly lower when compared with the control group. Proinflammatory mediators are also involved in the activation of osteoclasts (15). The suppression of proinflammatory mediator synthesis in the NC-2300 group may have induced down-regulation of both the synthesis and activation of osteoclasts, resulting in the limited expansion of periapical lesions in response to bacterial infection from infected root canals. Furthermore, NC-2300 may directly inhibit the action of osteoclasts. Cathepsin K is highly expressed in osteoclasts and is thought to play a key role in matrix degradation during bone resorption (12). Most of the cathepsin K inhibitors including NC-2300 target the action of cathepsin K in osteoclasts and reduce their bone-resorptive activity (20). It has been reported that knockdown of cathepsin K gene expression can significantly reduce bone destruction and cytokine expression in mouse periapical lesions (11).

The involvement of cathepsin K in proinflammatory cytokine synthesis has not yet been fully revealed; however, cathepsin K is reported to induce the activation of nuclear factor kappa B via mitogen-activated protein kinase signaling, such as extracellular signal-regulated kinase 1 and 2, and downstream of Toll-like receptor 9 in a rheumatoid arthritis model (13). In a psoriasis mouse model, NC-2300 exerts an antipsoriatic effect through down-regulation of the TLR7/IL-23/Th17 cascade in dendritic cells (21). We revealed that NC-2300 significantly down-regulates nitric oxide synthesis from LPS-stimulated macrophages, suggesting that cathepsin K may be involved in proinflammatory mediator synthesis from activated macrophages. The expression of cathepsin K in macrophages has been reported (22), and we also detected messenger RNA expression of cathepsin K in RAW264 macrophages *in vitro* (data

not shown). The expansion of periapical lesions correlates with the synthesis of proinflammatory cytokines from inflammatory cells, such as macrophages, as mentioned previously. The infiltration of inflammatory cells is typically observed in the expansion stage of periapical lesions (1, 2). NC-2300 down-regulated the infiltration of MHC class II molecule expressing cells, with most of them thought to be activated macrophages (2, 3). Various factors are involved in the infiltration of macrophages, and nitric oxide is one of the candidates for the induction of macrophage infiltration. The inhibition of inducible nitric oxide synthase, which is responsible for producing nitric oxide from macrophages, significantly down-regulates proinflammatory cytokine expression and the infiltration of inflammatory cells (23).

In summary, cathepsin K inhibitor administration effectively down-regulated periapical lesion expansion and synthesis of proinflammatory cytokines, suggesting that cathepsin K inhibitor could be used as an effective root canal medicament in the treatment of apical periodontitis.

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The authors deny any conflicts of interest related to this study.

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