# Anti-osteoclastogenesis of Mineral Trioxide Aggregate through Inhibition of the Autophagic Pathway

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## Abstract

**Introduction:** Mineral trioxide aggregate (MTA) regulates bone remodeling, particularly osteoclast differentiation. However, intracellular mechanisms underlying the anti-osteoclastogenesis of MTA remain unclear. This study aimed to evaluate the potential alterations of autophagic pathway during anti-osteoclastogenic effects by MTA *in vitro* and investigate their underlying mechanisms. **Methods:** Osteoclast precursors were treated with MTA extracts containing the receptor activator of nuclear factor-kappa B ligand (RANKL). Rapamycin was used to activate autophagy. RANKL-induced osteoclast differentiation was stained with tartrate-resistant acid phosphatase. Several specific autophagy features in osteoclast precursors were measured by using immunofluorescence, monodansylcadaverine, and transmission electron microscope. Autophagy-related proteins were investigated via Western blot analysis. The mRNA expression involved in autophagic and osteoclastic activities was detected with quantitative real-time polymerase chain reaction. **Results:** MTA extracts inhibited osteoclast differentiation via preventing the fusion of osteoclast precursors without cytotoxicity. MTA extracts interrupted RANKL-induced acidic vesicular organelle formation and autophagic vacuole appearance in osteoclast precursors. Moreover, autophagic genes and proteins stimulated with RANKL diminished with MTA extracts. Notably, autophagy activation through rapamycin promoted multinucleated osteoclasts formation and increased osteoclastic genes expression, which almost reversed MTA-mediated anti-osteoclastogenic effects. **Conclusions:** MTA inhibited osteoclastogenesis for bone repair through attenuating the autophagic pathway. *(J Endod 2017;■:1–8)*

## Key Words

Autophagy, microtubule-associated protein 1 light chain 3, mineral trioxide aggregate, osteoclastogenesis

### Significance

This study is the first to report that mineral trioxide aggregate (MTA) extracts suppressed RANKL-induced osteoclastogenesis for bone repair by inhibition of autophagic pathway. Moreover, targeting autophagic pathway can be a novel strategy for developing MTA-based modified materials.

Mineral trioxide aggregate (MTA) possesses excellent capacity to promote hard tissue regeneration. This material has been widely applied in endodontic repair therapies such as apexification (1), root-end filling (2), furcation perforation (3), and resorption (4). MTA leaches chemical components, including calcium and silica ions, on contact with tissue fluid (5). This phenomenon affects the biological activities of periapical cells such as dental pulp stem cells (6), periodontal ligament cells (7), and bone cells (8) in periapical tissue reconstruction. Mature osteoclasts derived from myeloid precursor cells perform secretion function to dissolve bone tissues during periapical periodontitis. It has been well-established that MTA extracts containing calcium and silica ions inhibit osteoclastogenesis (9) and bone resorption (10) to promote bone reconstruction. However, intracellular mechanisms involved in MTA-mediated anti-osteoclastogenic effects remain unclear.

Autophagy generally occurs in every cell that is critical for maintaining cellular homeostasis by recycling senescent organelles and damaged proteins. It participates in various cellular processes including cell death (11), pathogen clearance (12), and antigen presentation (13). As an adaptive response for survival, autophagy activity can be upregulated when cells are stimulated by nutrient deprivation, hypoxia, and radiation (14). The representative phenomenon during autophagy activity is the formation of double-membrane isolation structures enclosing damaged proteins and senescent organelles, which are known as autophagosomes. Subsequently, autophagosomes fuse with lysosomes in the cytoplasm to generate double-membrane autolysosomes, where the contents are digested by the acidic lysosome enzymes (15). Autophagy-related genes (Atg), namely Atg5-Atg12, Beclin 1, and microtubule-associated protein 1 light chain 3 (LC3), are key members to facilitate autophagy-related membrane structures formation and function. The transformation of LC3-I into LC3-II, the 2 forms of LC3, is considered the pivotal process during autophagy activity (16).

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Studies demonstrated that autophagy is a critical regulator of osteoclast differentiation and function (17). Autophagic proteins such as Atg5, Atg7, and Atg4B are essential for ruffled-border formation and secretory function of osteoclasts involved in bone resorption (18, 19). Moreover, LC3 promotes actin-ring formation and cathepsin K (CatK) release, which enhance bone resorption (20). Nevertheless, the association between MTA-mediated anti-osteoclastogenesis and autophagy remains unclear. In the present study, we hypothesized that MTA-elicited anti-osteoclastogenesis was through inhibition of the autophagic pathway.

RAW 264.7 cells are a murine macrophage cell line that can differentiate into mature osteoclasts under the stimulation of the receptor activator of nuclear factor-kappa B ligand (RANKL) (9, 21). This study aimed to investigate the potential role of the autophagic pathway in MTA-elicited anti-osteoclastogenic effects.

## Materials and Methods

### Material Preparation

ProRoot MTA (Dentsply, Tulsa, OK) was mixed with distilled water under sterile conditions as previously described (9, 22). The cement was shaped in cylindrical plastic molds (diameter, 11 mm; height, 4 mm). Subsequently, the specimens were incubated under the humidified condition of 37°C for 1 week. After completely set, the specimens were immersed in 10 mL $\alpha$-minimal essential medium ($\alpha$-MEM) (Gibco-BRL, Grand Island, NY) for 4 days at 37°C under 100% relative humidity. The medium was then sterilized by using a 0.22 $\mu$m filter. Several concentrations (undiluted, one half, and one fourth dilutions) of MTA extracts were prepared for the subsequent experiments.

### Cell Culture

RAW 264.7 cells (China Type Culture Collection) were maintained in $\alpha$-MEM containing 50 ng/mL RANKL (Peprotech, London, United Kingdom) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in an incubator humidified with 5% $CO_2$.

### Cytotoxicity Assay

RAW 264.7 cells were plated ($8 \times 10^3$ cells/well) onto 96-well plates. After attachment, the cells were stimulated with serially diluted MTA extracts for the indicated time (24, 48, or 72 hours). Cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was performed following the manufacturer's protocol. The reagent (from CCK-8) mixed with $\alpha$-MEM (1:10) was added before incubation at 37°C for another 2 hours. Detection of optical density at 450 nm was acquired by using an enzyme-linked immunosorbent assay microplate reader (Bio-Tek Instruments, Winooski, VT).

### Tartrate-resistant Acid Phosphatase Staining

RAW 264.7 cells were stimulated in serially diluted MTA extracts containing 50 ng/mL RANKL for 5 days with or without rapamycin pretreatment. The cells were fixed with 4% paraformaldehyde for 15 minutes and stained by using leukocyte acid phosphatase assay kit (Sigma-Aldrich, St Louis, MO) following the manufacturer's protocol. Purple multinucleated cells with 3 or more nuclei under optical microscope were marked as mature osteoclasts and counted. The results are regarded as positive for tartrate-resistant acid phosphatase (TRAP) staining.

### Monodansylcadaverine

After 12 hours of incubation with undiluted MTA extracts containing RANKL (50 ng/mL) with or without rapamycin pretreatment, the cells were incubated in monodansylcadaverine (MDC) (50 $\mu$mol/L) (Sigma-Aldrich) protected from light for 15 minutes. Then acidic vesicular organelle (AVO)-labeled green fluorescence in the cytoplasm was immediately visualized under a fluorescence microscope (Leica, Wetzlar, Germany).

### Double Immunofluorescence Labeling and Fibrous Actin Formation Assay

RAW 264.7 cells were grown on glass coverslips. After incubation (6 hours or 5 days) with undiluted MTA extracts containing RANKL (50 ng/mL) with or without rapamycin pretreatment, the cells were fixed and permeabilized with 0.1% Triton–phosphate-buffered saline for 10 minutes. Ten percent goat serum was used to block the cells at 37°C for 1 hour and incubated with rabbit antibody of LC3-II (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 24 hours. The primary antibody adequately combined with red fluorescently labeled goat anti-rabbit immunoglobulin G (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 1 hour, and fibrous actin (F-actin) rings were labeled with fluorescein isothiocyanate–conjugated phalloidin (Sigma-Aldrich) at 37°C for 40 minutes. After thoroughly washing, the nuclei were fluorescence labeled by using 4′,6-diamidino-2-phenylindole at 37°C for 5 minutes. Finally, fluorescent images were viewed under a fluorescence microscope (Leica).

### Transmission Electron Microscopy

RAW 264.7 cells were cultured with undiluted MTA extracts containing RANKL (50 ng/mL) in 6-cm dish for 6 hours. The cells were handled as described previously (21). The ultrastructure of autophagosomes or autolysosomes was determined by using a transmission electron microscope (Hitachi, Tokyo, Japan).

### Western Blot Analysis

RAW 264.7 cells were pretreated with or without rapamycin for 4 hours. Then the cells were maintained with undiluted MTA extracts containing RANKL (50 ng/mL) with or without rapamycin pretreatment for 6 hours. The cells were lysed on ice for 5 minutes. All extracted proteins were obtained from the suspension via centrifugation (15,000*g*, 15 minutes). All extracts were quantitated with a BCA protein determination method (Applygen Technologies Inc, Beijing, China). Western blot analysis was performed by using the protein extracts according to a published report (23). The following specific primary antibodies were used: Beclin 1 (1:1000 dilution; Proteintech, Chicago, IL), Atg5-Atg12 (1:1000 dilution; Proteintech), and LC3-II (1:1000 dilution; Santa Cruz Biotechnology). Beta-actin (1:5000 dilution; Proteintech) was regarded as the loading control.

**TABLE 1.** Specific Primer Sequences for RT-PCR

| Gene name | Forward sequences | Reverse sequences |
|---|---|---|
| GAPDH | 5′-CTCCCACTCTTCCACCTTCG-3′ | 5′-TTGCTGTAGCCGTATTCATT-3′ |
| CatK | 5′-CAGCAGAACGGAGGCATTGA-3′ | 5′-CCTTTGCCGTGGCGTTATAC-3′ |
| Atg5-Atg12 | 5′-GCCTATATGTACTGCTTCATCCA-3′ | 5′-CATTTCAGGGGTGTGCCTTCA-3′ |
| LC3-II | 5′-CGGAGCTTTGAACAAAGAGTG-3′ | 5′-TCTCTCACTCTCGTACACTTC-3′ |
| MMP-9 | 5′-TCCAGTACCAAGACAAAGCCTA-3′ | 5′-TTGCACTGCACGGTTGAA-3′ |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

 