Title: Crystalline structure of pulverized dental calculus induces cell death in oral epithelial cells

Short title: Epithelial cell death induced by dental calculus

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Abstract

Background and Objective: Dental calculus is a mineralized deposit attached to the tooth surface. We have shown that cellular uptake of dental calculus triggers nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activation, leading to the processing of the interleukin-1β precursor into its mature form in mouse and human phagocytes. The activation of the NLRP3 inflammasome also induced a lytic form of programmed cell death, pyroptosis, in these cells. However, the effects of dental calculus on other cell types in periodontal tissue have not been investigated. The aim of this study was to determine whether dental calculus can induce cell death in oral epithelial cells.

Material and Methods: HSC-2 human oral squamous carcinoma cells, HOMK107 human primary oral epithelial cells and immortalized mouse macrophages were exposed to dental calculus or one of its components, hydroxyapatite crystals. For inhibition assays, the cells were exposed to dental calculus in the presence or absence of cytochalasin D (endocytosis inhibitor), z-YVAD-fmk (caspase-1 inhibitor) or glyburide (NLRP3 inflammasome inhibitor). Cytotoxicity was determined by measuring lactate

dehydrogenase (LDH) release and staining with propidium iodide (PI). Tumor necrosis factor (TNF)-α production was quantified by enzyme-linked immunosorbent assay. Oral epithelial barrier function was examined by permeability assay.

Results: Dental calculus induced cell death in HSC-2 cells, as judged by LDH release and PI staining. Dental calculus also induced LDH release from HOMK107 cells. Following heat treatment, dental calculus lost its capacity to induce TNF-α in mouse macrophages, but could induce LDH release in HSC-2 cells, indicating a major role of inorganic components in cell death. Hydroxyapatite crystals also induced cell death in both HSC-2 and HOMK107 cells, as judged by LDH release, indicating the capacity of crystal particles to induce cell death. Cell death induced by dental calculus was significantly inhibited by cytochalasin D, z-YVAD-fmk and glyburide, indicating NLRP3 inflammasome involvement. In permeability assays, dental calculus attenuated the barrier function of HSC-2 cell monolayers.

Conclusions: Dental calculus induces pyroptotic cell death in human oral epithelial cells and the crystalline structure plays a major role in this process. Oral epithelial cell death induced by dental calculus might be important for the etiology of periodontitis.

Introduction

Dental calculus is a mineralized deposit frequently found on the tooth surfaces of periodontitis patients. Dental calculus formation is usually preceded by the development of dental plaque.[1] Mineralization is initiated by the binding of calcium ions to carbohydrate-protein complexes of the organic matrix, followed by precipitation of crystalline calcium phosphate salts in the intercellular matrix of the bacteria. Electron microscopy of dental calculus has demonstrated the presence of minute crystals, 500–4000 Å long, deposited on mineralized microorganisms.[2] Studies have shown strong correlations between calculus and periodontitis and the effectiveness of calculus removal on improvements of periodontal conditions.[1]

We recently showed that dental calculus induces interleukin (IL)-1β secretion and cell death by activating nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in human and mouse phagocytes.[3] However, the effects of dental calculus on other cell types in periodontal tissue have not been investigated. The crevicular/pocket epithelium is located anatomically closest to dental calculus in periodontal tissue. These epithelia act as the first line of defense

against invading bacteria and other external stimuli, protecting periodontal tissue as a physical barrier.[4,5] While epithelial cells are not an authentic inflammatory cell type and cannot be the dominant source of IL-1β,[6] they express NLRP3 inflammasome components.[7,8] Upon activation of NLRP3 inflammasome, caspase-1 cleaves a 53 kDa substrate called gasdermin D, and the N-terminal fragment of gasdermin D assembles into pores in the cell membrane which causes cell death, pyroptosis.[9] Therefore, we hypothesized that dental calculus may induce cell death via the NLRP3 inflammasome activation in oral epithelial cells and modify the barrier function of the crevicular/pocket epithelium, which is generally observed at diseased sites.[10]

This study aimed to determine whether dental calculus can induce cell death via the NLRP3 inflammasome activation in oral epithelial cells. We also examined the role of the crystalline structure using synthetic hydroxyapatite (HA) crystals, one of the main components of dental calculus, in oral epithelial cell death.

Materials and Methods

Dental Calculus

Dental calculus samples were obtained from five chronic periodontitis patients who visited the Nagasaki University Hospital. Written informed consent was obtained from all participants. The study design and procedure for obtaining informed consent were approved by the Institutional Review Board at Nagasaki University (No. 1394-2). Each dental calculus sample was treated as described previously.[3] Briefly, samples were collected using sterile Gracey curettes, pulverized with a mortar and pestle for 15 min, washed with 10% sodium hypochlorite and distilled water, filtered using a 48 μm pore-sized nylon mesh, and autoclaved. To analyze the size distribution, pulverized dental calculi were dusted onto conductive carbon tape, coated with platinum-palladium and observed by scanning electron microscopy using a Hitachi tabletop microscope TM1000 (Hitachi High-Technologies, Tokyo, Japan). Data were analyzed based on ten scanned images using ImageJ software **(**National Institutes of Health, Bethesda, Maryland). Some calculi were treated at 250°C for 1 h to remove microbial remnants,[11]

termed “baked calculus”. This heat treatment has been reported to effectively destroy pyrogenic substances, such as endotoxin.[12] All prepared calculus samples were adjusted to the appropriate concentrations and vigorously vortexed before use.

Reagents

Dulbecco’s modified Eagle medium (DMEM), Opti-MEM, Epilife basal medium, phosphate-buffered saline (PBS), penicillin-streptomycin, and Epilife defined growth supplement (EDGS) were purchased from Thermo Fisher Scientific (Waltham, MA). Fetal bovine serum (FBS) was obtained from Equitec-Bio (Kerrville, TX). Synthetic HA crystals, ≤ 5 μm in diameter, were purchased from SofSera (Tokyo, Japan). Minimum essential medium eagle (MEM), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), propidium iodide (PI), necrostatin-1, and glyburide were purchased from Sigma-Aldrich (St. Louis, MO). z-YVAD-fmk was obtained from Calbiochem-EMD Millipore (Darmstadt, Germany). Caspase-1 assay kit and CA-074Me were purchased from Enzo Life Sciences (Farmingdale, NY). Cytochalasin D and trypan blue were purchased from Wako Pure Chemical Industries (Osaka, Japan).

A mouse tumor necrosis factor (TNF)-α DuoSet enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). A CytoTox 96 Non-Radioactive Cytotoxicity Assay was purchased from Promega (Madison, WI). Ultra-pure LPS from *Escherichia coli* O111:B4 and synthetic triacylated lipopeptide, Pam_3CSK_4, were obtained from InvivoGen (San Diego, CA). Bio-Rad Protein Assay was purchased from Bio-Rad (Hercules, CA).

Cell Culture

HSC-2 cells derived from human oral squamous cell carcinoma were obtained from the Riken Cell Bank (Tsukuba, Japan) and cultured in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HOMK107 cells obtained via a surgical procedure using the explant technique from human gingiva were purchased from Cell Research Corporation (Singapore) and cultured in Epilife basal medium supplemented with 1% EDGS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HOMK107 cells were only used for experiments within four passages due to their

limited life span. Immortalized C57BL/6 mouse bone marrow macrophages were obtained and cultured as previously described.[3]

Cytotoxicity Assay

HSC-2 or HOMK107 (2×10^4) cells were seeded in 96-well plates and exposed to various concentrations of dental calculus (unbaked calculus, unless otherwise indicated) or HA crystals in the presence of 0.5% FBS (HSC-2 cells only) for 24 h. For inhibition assays, the cells were pre-incubated with cytochalasin D (actin polymerization inhibitor), z-YVAD-fmk (caspase-1 inhibitor), glyburide (NLRP3 inhibitor, ATP-sensitive K^+ channel blocker), Ca-074Me (cathepsin B inhibitor), or necrostatin-1 (receptor-interacting protein kinase 1 inhibitor) for 30 min. To analyze the effects of microbial components, HSC-2 cells were pre-incubated with ultra-pure LPS from *E. coli* O111:B4 or Pam_3CSK_4 for 2 h. After incubation, the plates were centrifuged and cell culture supernatants were harvested. Lactate dehydrogenase (LDH) in the supernatants was then measured using a CytoTox 96 Non-Radioactive assay according

to the manufacturer's protocol. Percent cytotoxicity was determined according to the following calculation[13]:

% Cytotoxicity = 100 × [Experimental LDH release (OD) – Background LDH release (OD)]/[Maximum LDH release (OD) – Background LDH release (OD)].

PI staining

HSC-2 (3×10^5) cells were seeded in 35 mm dishes and incubated for 24 h. Then, the cells were exposed to PBS, unbaked calculus, or HA crystals for 24 h. As a positive control, HSC-2 cells were incubated at -80°C for 30 min and thawed. The cells were then stained with PI (100 μg/mL) and incubated in the dark for 15 min. Images of PI positive cells were acquired using a fluorescence microscope (Zeiss, Axiovert 200M, Oberkochen, Germany) with a Texas Red filter. The numbers of PI-positive cells were counted using ImageJ software.

TNF-α measurement

Immortalized mouse macrophages (1×10^5) were seeded in 96-well plates, then stimulated with unbaked calculus, baked calculus, or HA crystals for 6 h. The culture supernatants were harvested and concentrations of TNF-α were quantified by ELISA, according to the manufacturer's protocol.

Caspase-1 detection assay

To determine caspase-1 activity, HSC-2 cells were exposed to various concentrations of dental calculus for 24 h. After incubation, the cells were lysed by three cycles of freezing and thawing and centrifuged at 10, 000 g for 1 min. The cell lysates were concentrated and their protein concentrations were measured using Bio-Rad Protein Assay. The caspase-1 activity was calculated from the cleavage of a colorimetric substrate, Ac-YVAD-pNA, using the caspase-1 assay kit. Substrate cleavage was determined with the absorbance at 405 nm wavelength using a microplate reader. The data were calculated as enzymatic units/mg of total protein.

Permeability Assay

The permeability assay was performed following the previously described procedure with slight modifications.[14] HSC-2 (2×10^5) cells were incubated in the upper insert of a transwell membrane (Corning, NY). After the formation of a compact monolayer, the cells were exposed to dental calculus or HA crystals for 24 h. The medium in the upper insert was then replaced with 200 μL of Opti-MEM containing 1% BSA and 0.5% trypan blue, and the medium in the lower well was replaced with fresh Opti-MEM. After a 1 h incubation, the medium in the lower well was collected and optical absorbance was measured at 610 nm using an automated plate reader. From the absorbance, triplicates of the average concentration of trypan blue that had passed through the cell monolayer were calculated from a standard curve.

Statistical Analysis

Statistical differences between the test and control groups were analyzed by t-test using Stat Mate 4 (ATMS, Tokyo, Japan).

Results

Dental calculus induces cell death in HSC-2 cells

The sizes of pulverized dental calculus particles were measured using scanning electron microscope images (Figure 1A) and results showed that more than 90% of the particles ranged from 0.1 to 10 μm (Figure 1B). To explore the capacity of dental calculus to induce cell death, HSC-2 human oral squamous carcinoma cells were exposed to increasing concentrations of dental calculus or were left untreated for 24 h. Dental calculus induced dose- and time-dependent cell death as judged by LDH released from HSC-2 cells (Figure 2A, B). To confirm cell death, HSC-2 cells were exposed to 500 μg/ml of dental calculus for 24 h and stained with PI. Considerable numbers of cells were stained with PI following exposure to dental calculus, whereas few untreated cells were stained (Figure 2C). The number of PI-positive cells correlated with the concentration of dental calculus (Figure 2D).

Crystalline structure in dental calculus contributes to cell death in HSC-2 cells

To eliminate the possible effects of microbial ligands on cell death in HSC-2 cells,[15, 16] we baked the calculus from five periodontitis patients, and exposed HSC-2 cells to baked or unbaked calculus. Each unbaked calculus sample induced cell death in HSC-2 cells, and similar levels of cell death were also induced by the corresponding baked calculus sample (Figure 3A), suggesting that cell death was mainly induced by inorganic components of the calculus. To confirm the heat-treatment effect, immortalized mouse macrophages were exposed to baked or unbaked calculus. Levels of TNF-α induced by the baked calculus samples were significantly lower compared with those induced by the corresponding unbaked calculus samples (Figure 3B).

To further examine the possible effects of soluble factors, we exposed HSC-2 cells to dental calculus in the presence or absence of an actin-polymerization inhibitor, cytochalasin D, for 24 h. Cytochalasin D attenuated cytotoxicity, indicating the necessity of endocytosis for cell death (Figure 3C). To investigate the role of the crystalline structure of dental calculus, we exposed HSC-2 cells to synthetic HA crystals, one of the main crystal forms comprising dental calculus. HA particles induced dose- and time-dependent cell death in HSC-2 cells as judged by LDH release (Figure 4A, B).

When HSC-2 cells were stained with PI following exposure to HA particles for 24 h, a considerable number of PI-positive cells were detected (Figure 4C). The number of PI-positive cells correlated with the concentration of HA crystals (Figure 4D). HA particles did not induce TNF-α production in immortalized mouse macrophages, indicating no contamination of the microbial stimuli (Figure 5A). Cell death induced by HA particles was inhibited by cytochalasin D, confirming the necessity of endocytosis for cell death (Figure 5B). To further examine the role of bacterial components in cell death, HSC-2 cells were exposed to 250 μg/ml of HA particles in the presence or absence of LPS or Pam_3CSK_4. Neither LPS nor Pam_3CSK_4 induced significant increases in cytotoxicity (Figure 6).

Dental calculus induces NLRP3 inflammasome-dependent cell death in HSC-2 cells

Studies have shown that various crystals induce pyroptosis through activation of the NLRP3 inflammasome in macrophages,[17,18] while another has shown that crystals induce necroptosis through the RIP kinase pathway in renal progenitor cells.[19] First, we examined if dental calculus activates caspase-1 in HSC-2 cells. As shown in Figure 7A,

the caspase-1 activity correlated with the concentration of dental calculus, confirming the activation of inflammasome by dental calculus. Then, HSC-2 cells were exposed to dental calculus in the presence or absence of z-YVAD-fmk or glyburide. The inhibitors partially attenuated cell death in HSC-2 cells, indicating NLRP3 inflammasome involvement (Figure 7B, C). Endocytosis of crystal particles damages the lysosome membrane, and inhibition of cathepsin B, a lysosomal protease, has been shown to suppress NLRP3 activation.[20, 21] Therefore, HSC-2 cells were exposed to dental calculus in the presence of Ca-074Me, a chemical inhibitor of cathepsin B, and cell death was partially inhibited by Ca-074Me (Figure 7D). In contrast to these inflammasome inhibitors, necrostatin-1 did not inhibit cell death in HSC-2 cells induced by dental calculus, indicating no or weak involvement of necroptosis (Figure 7E).

Dental calculus induces cell death in primary oral epithelial cells and decreases the barrier function of the HSC-2 cell monolayer

To investigate whether stimulation with dental calculus leads to cell death in primary oral epithelial cells, HOMK107 cells derived from human gingiva were stimulated with increasing doses of dental calculus for 24 h. Dental calculus induced

cell death in HOMK107 cells as judged by LDH release (Figure 8A). Similarly, HA crystals induced cell death in HOMK107 cells (Figure 8B).

To examine the effects of dental calculus on the barrier function of HSC-2 monolayers, permeability assays were performed. The amount of pigment passing through the HSC-2 cell monolayer significantly increased following exposure to dental calculus or HA crystals, compared with controls (Figure 8C).

Discussion

Dental calculus induced LDH release from HSC-2 oral squamous carcinoma cells in a dose- and time-dependent manner. Cell death in HSC-2 cells was confirmed by PI staining following exposure to dental calculus. The capacity of dental calculus to induce cell death was also examined using HOMK107 primary gingival epithelial cells. To our knowledge, this is the first evidence showing the capacity of dental calculus to induce oral epithelial cell death.

Although dental calculus was extensively washed with 10% sodium hypochlorite and distilled water, it was difficult to remove all the bacterial remnants that may trigger cell death in oral epithelial cells.[15] In fact, unbaked calculus induced TNF-α in immortalized mouse macrophages, indicating the presence of microbial ligands. To inactivate these, dental calculus was treated at 250°C for 1 h. This heat-treatment successfully diminished the signaling necessary for TNF-α production, but failed to reduce the capacity of dental calculus to induce cell death in HSC-2 cells. This is in clear contrast with our previous study showing that heat treatment abrogated the capacity of calculus to induce IL-1β in mouse macrophages.[3] These results suggest that

the induction of cell death in oral epithelial cells was mainly mediated by the inorganic components of dental calculus and that cell death could be induced without priming by microbial ligands. The importance of inorganic components is also supported by the fact that synthetic HA crystals, which did not contain any microbial stimuli as indicated by TNF-α production in mouse macrophages, induced cell death in HSC-2 and HOMK107 cells. Furthermore, pre-incubation of HSC-2 cells with LPS or Pam_3CSK_4 resulted in no significant cytotoxicity increases. Together, these results suggest that the crystalline structure of dental calculus plays a major role in the cell death of oral epithelial cells exposed to dental calculus. The non-significant contribution of microbial products to cell death might be due to the low sensitivity of oral epithelial cells to microbial ligands. Studies have shown that oral keratinocytes do not express CD14 and lipopolysaccharide-binding protein and have low expression of Toll-like receptor 4.[22] The limited expression of these pattern recognition receptors might contribute to their low sensitivity to microbial ligands. However, live bacteria in dental plaque may play more important roles in epithelial cell death than bacterial remnants in dental calculus.[8,16]

Research has shown that crystal particles, such as silica and MSU, induce pyroptosis through NLRP3 inflammasome activation in various epithelial cells.[17,18] When HSC-2 cells were exposed to dental calculus in the presence of caspase-1, NLRP3, or cathepsin B inhibitors, cell death was partially inhibited. These results indicate that NLRP3 inflammasome activation was at least partially involved in calculus-induced cell death in HSC-2 cells. Although one study has shown that various crystals, such as calcium oxalate, MSU, and calcium pyrophosphate dehydrate induce necroptosis in renal tubular cells,[19] a RIPK-1 inhibitor, necrostatin-1, did not inhibit cell death induced by dental calculus in HSC-2 cells. The weak or no involvement of necroptosis might be due to the nature of the original tissue.

In addition to a variety of bacterial ligands in dental plaque that can induce cell death,[8,16] the present findings revealed that dental calculus also induces cell death in oral epithelial cells. When the HSC-2 cell monolayer was exposed to dental calculus or HA crystals, its permeability significantly increased. These findings suggest that dental calculus can deteriorate the barrier function of crevicular/pocket epithelium. Higher permeability may cause greater infiltration of bacteria and inflammatory cells. Persistent

inflammation may therefore occur because crystal particles cannot be digested or removed by immune cells.[23] The relative importance of bacteria and dental calculus to epithelial barrier function alterations should be determined in an *in vivo* study.

In the present study, we showed the capacity of dental calculus to induce cell death in oral epithelial cells. Previous analyses of dental calculus by electron microscopy demonstrated the presence of various sizes of crystals, such as 50–400 nm small crystals in the intermicrobial spaces, 0.1-1.5 μm calcified microorganisms and 5-20 μm calcified microcolonies.[2,25] These particles are densely deposited on the outer layers of deep subgingival calculus,[24,25] and their sizes are in the same range with the pulverized dental calculus used in this study. It is plausible that these small calculus particles induce cell death in crevicular/pocket epithelium. Unlike the induction of IL-1β, priming by microbial ligands was not mandatory to induce cell death by dental calculus. Thus, dental calculus might be one of the major causes of alterations in crevicular/pocket epithelium permeability. Considering these effects, dentists should not leave subgingival plaque until it begins to calcify; a certain amount of subgingival deposits should be removed as part of routine periodontal therapy, even when no

periodontal disease activity is detected. New strategies to prevent and eliminate dental calculus should be developed.

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Figure legends

Figure 1

Morphological analysis of pulverized dental calculus.

(A) Scanning electron microscope images of pulverized dental calculus particles. The white bar represents 10 μm. (B) The size distribution of dental calculus particles.

Figure 2

Dental calculus induces cell death in a dose- and time-dependent manner in HSC-2 cells.

HSC-2 cells were exposed to the indicated concentrations of (A, C, D) dental calculus for 24 h, or (B) to 500 μg/mL of dental calculus for the indicated time. (A, B) Cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. (C) The cells were also stained with propidium iodide (PI) and the number of stained cells are shown (D). The results were quantified from at least three independent experiments and are expressed as the mean ± standard error of triplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (t-test).

Figure 3

Effects of microbial remnants in dental calculus on cell death in HSC-2 cells and TNF-α production in mouse macrophages.

(A) Dental calculus samples obtained from five periodontitis patients were treated at 250°C for 1 h (baked) or left untreated (unbaked). HSC-2 cells were exposed to 500 μg/mL of unbaked or baked calculus for 24 h, and cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. Averages of three independent experiments are shown. (B) Immortalized mouse macrophages were stimulated with 500 μg/mL of unbaked or baked calculus for 6 h. TNF-α concentrations in the culture supernatants were measured by ELISA. Representative results from three independent experiments are shown. (C) HSC-2 cells were exposed to 500 μg/mL of dental calculus in the presence of the indicated concentrations of the endocytosis inhibitor, cytochalasin D. After 24 h of incubation, cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. Representative results from three independent experiments are shown. The results are expressed as the mean ± standard error of triplicate assays. **$p < 0.01$; ***$p < 0.001$ (t-test).

Figure 4

HA crystals induce cell death in a dose- and time-dependent manner in HSC-2 cells.

HSC-2 cell were exposed to the indicated concentration of (A, C, D) hydroxyapatite (HA) crystals for 24 h or (B) to 500 μg/mL of HA crystals for the indicated time. (A, B) Cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. (C) The cells were also stained with PI and the numbers of stained cells are shown (D). Representative results from three independent experiments are shown. The results are expressed as the mean ± standard error of triplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (t-test).

Figure 5

HA crystals induce endocytosis-mediated cell death in HSC-2 cells but not TNF-α production in mouse macrophages.

(A) Immortalized mouse macrophages were stimulated with 500 μg/mL of unbaked calculus or HA crystals for 6 h. TNF-α concentrations in the culture supernatants were measured by ELISA. (B) HSC-2 cells were exposed to 500 μg/mL of HA crystals in the

presence of the indicated concentrations of cytochalasin D. After 24 h of incubation, cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. Representative results from three independent experiments are shown. The results are expressed as the mean ± standard error of triplicate assays. **$p < 0.01$; ***$p < 0.001$ (t-test).

Figure 6

Effects of microbial components on HA crystal-induced cell death in HSC-2 cells.

HSC-2 cells were exposed to 250 μg/mL of HA crystals in the presence or absence of the indicated concentrations of (A) LPS or (B) Pam_3CSK_4. After a 24 h incubation, cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. Representative results from three independent experiments are shown. The results are expressed as the mean ± standard error of triplicate assays.

Figure 7

Effects of caspase-1, NLRP3 inflammasome, cathepsin B, and RIPK1 inhibitors on cell death induced by dental calculus.

(A) HSC-2 cells were exposed to the indicated concentrations of dental calculus for 24 h and the caspase-1 activity in the cell lysates were measured as described in the Materials and Methods. (B-E) HSC-2 cells were exposed to 500 μg/mL of dental calculus in the presence of the indicated concentrations of (B) the caspase-1 inhibitor, z-YVAD-fmk, (C) the NLRP3 inflammasome inhibitor, glyburide, (D) the cathepsin B inhibitor, Ca-074Me, or (E) the RIPK1 inhibitor, necrostatin-1 for 24 h. Cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. Representative results from three independent experiments are shown. The results are expressed as the mean ± standard error of triplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (t-test).

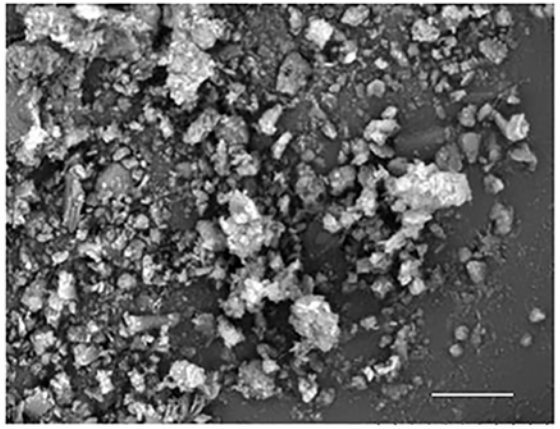
Figure 8

Dental calculus and HA crystals induce cell death in primary epithelial cells and alter the permeability of the HSC-2 cell lining.

HOMK107 epithelial cells derived from human oral mucosa were exposed to the indicated concentrations of (A) dental calculus or (B) HA crystals for 24 h. Cytotoxicity was quantified by measuring LDH concentrations in the culture supernatants. (C) The HSC-2 cell monolayer was left unexposed or was exposed to 500 μg/mL of dental calculus or HA crystals for 24 h. Relative changes in permeability were determined by measuring the trypan blue pigments that passed through HSC-2 cell monolayer formed on the membrane. Representative results from three independent experiments are shown. The results were expressed as the mean ± standard error of triplicate assays. *$p < 0.05$; **$p < 0.01$ (t-test).

Figure 1

(A)



(B)

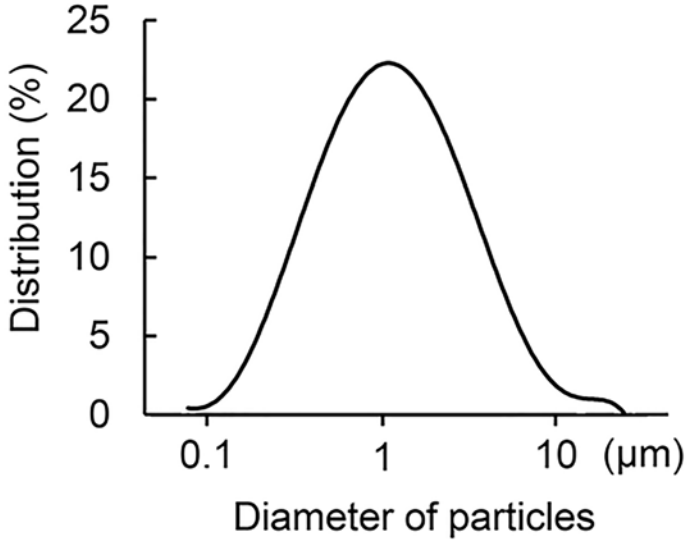


Figure 2

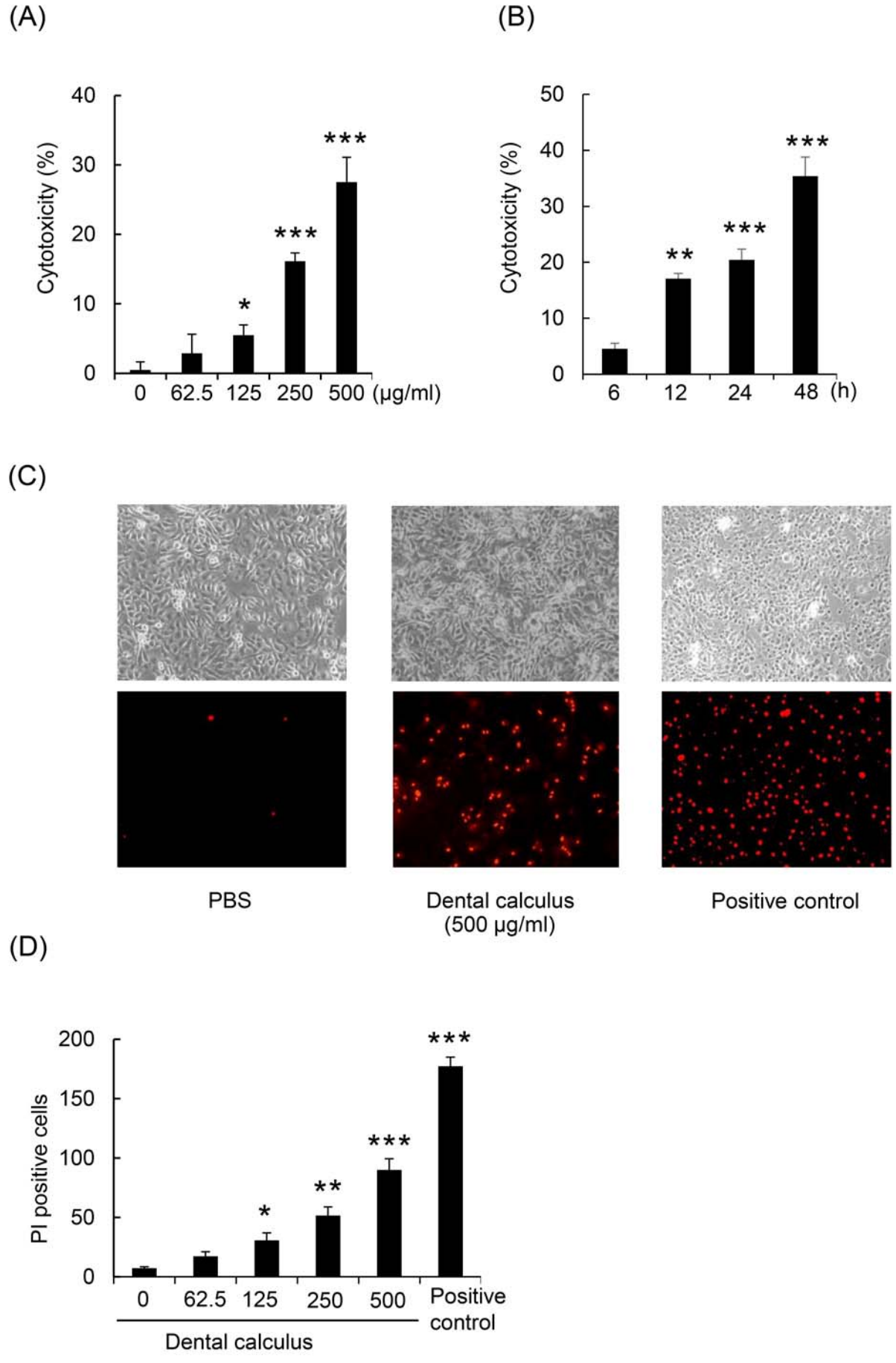

(A)
Cytotoxicity (%)
40
30
20
10
0
*
***
***
0
62.5
125
250
500
(μg/ml)
(B)
Cytotoxicity (%)
50
40
30
20
10
0
**
***
***
6
12
24
48
(h)
(C)
PBS
Dental calculus
(500 μg/ml)
Positive control
(D)
PI positive cells
200
150
100
50
0
*
**
***
***
0
62.5
125
250
500
Positive
control
Dental calculus

Figure 3

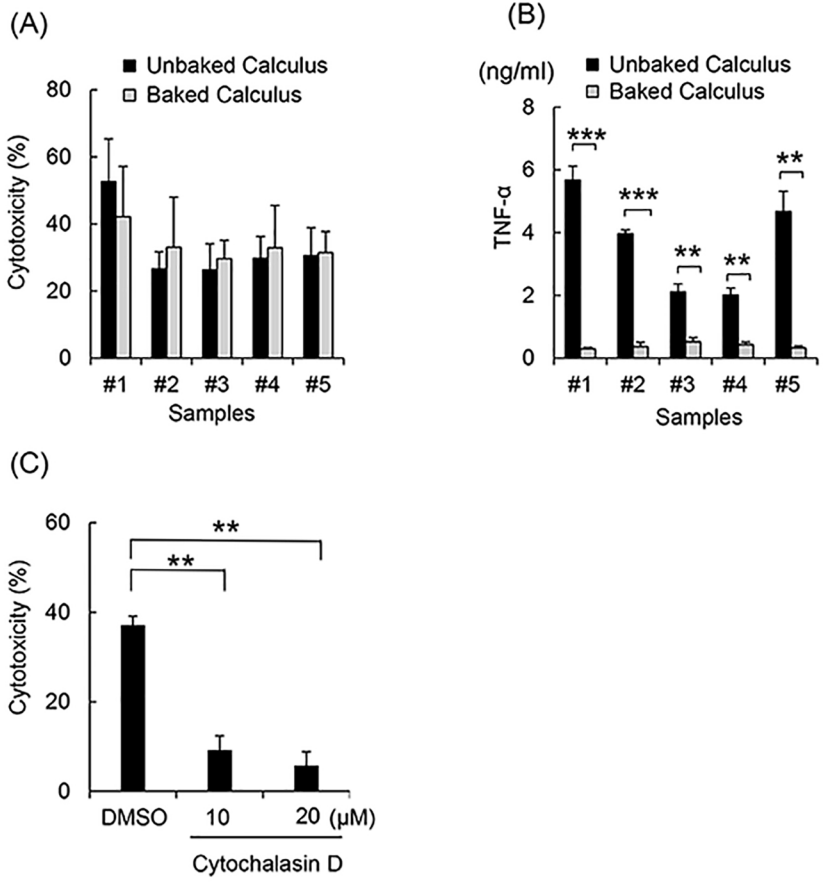

(A)
Unbaked Calculus
Baked Calculus
Cytotoxicity (%)
80
60
40
20
0
#1
#2
#3
#4
#5
Samples
(B)
(ng/ml)
Unbaked Calculus
Baked Calculus
TNF-α
8
6
4
2
0
***
***
**
**
**
#1
#2
#3
#4
#5
Samples
(C)
Cytotoxicity (%)
60
40
20
0
**
**
DMSO
10
20 (μM)
Cytochalasin D

Figure 4

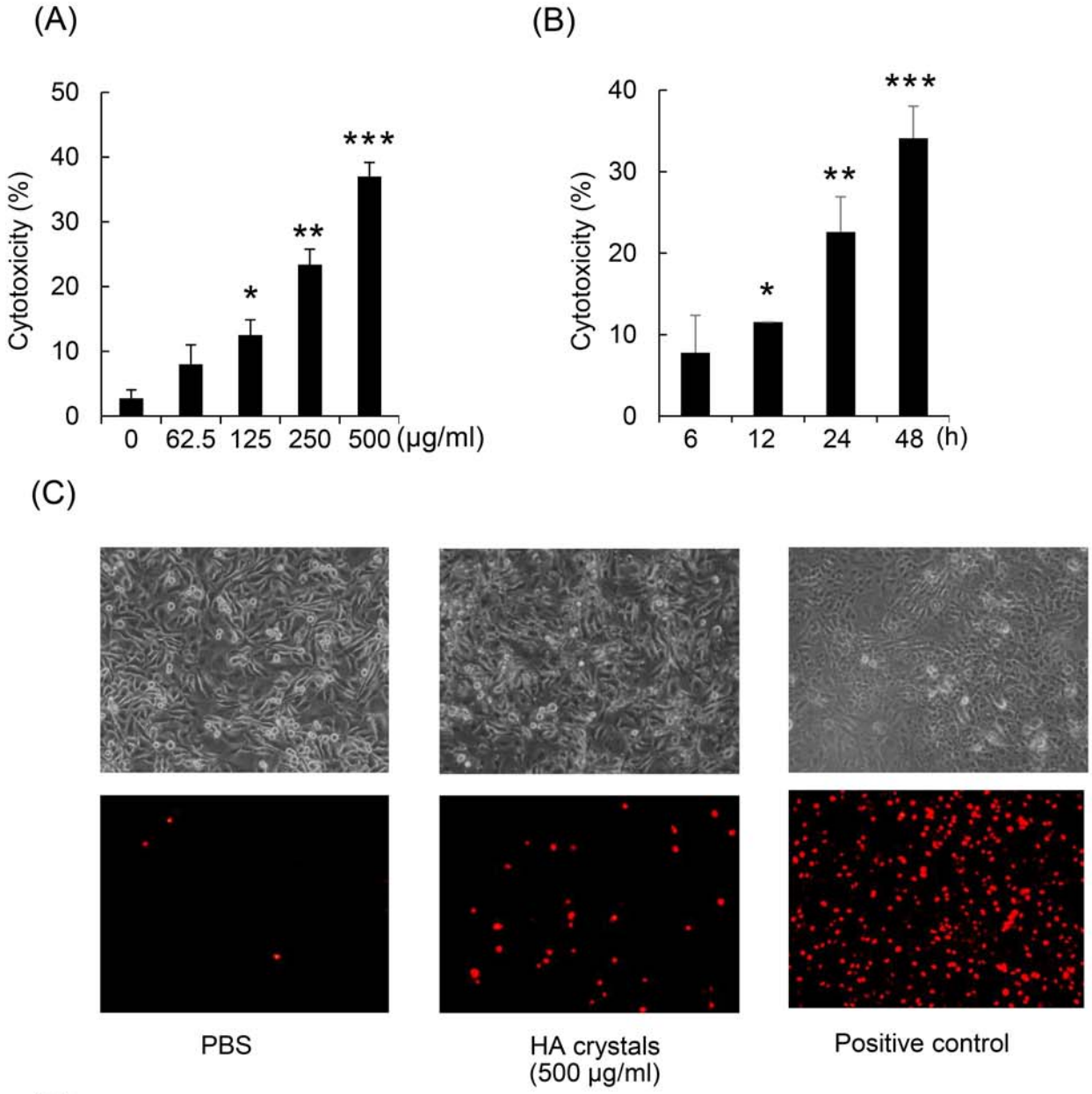

(A)
Cytotoxicity (%)
50
40
30
20
10
0
*
**
***
0
62.5
125
250
500
(μg/ml)
(B)
Cytotoxicity (%)
40
30
20
10
0
*
**
***
6
12
24
48
(h)
(C)
PBS
HA crystals
(500 μg/ml)
Positive control
(D)
PI positive cells
450
300
150
0
*
*
*
***
***
0
62.5
125
250
500
Positive
control
HA crystals

Figure 5

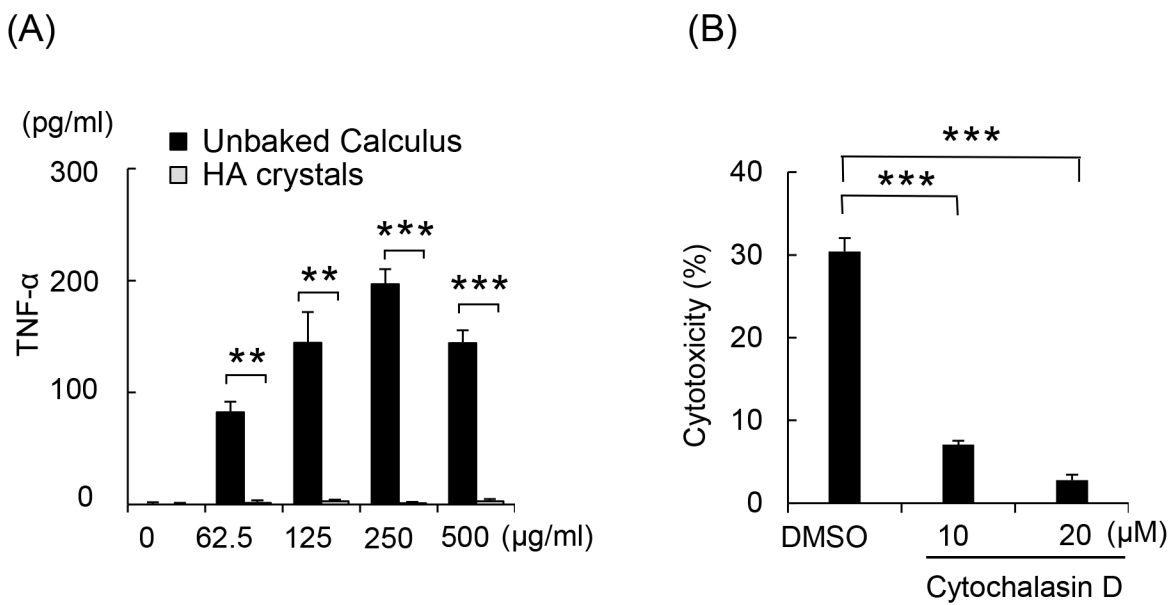

(A)
(pg/ml)
Unbaked Calculus
HA crystals
300
200
100
0
TNF-α
**
**
***
***
0
62.5
125
250
500
(μg/ml)
(B)
***
***
40
30
20
10
0
Cytotoxicity (%)
DMSO
10
20
(μM)
Cytochalasin D

Figure 6

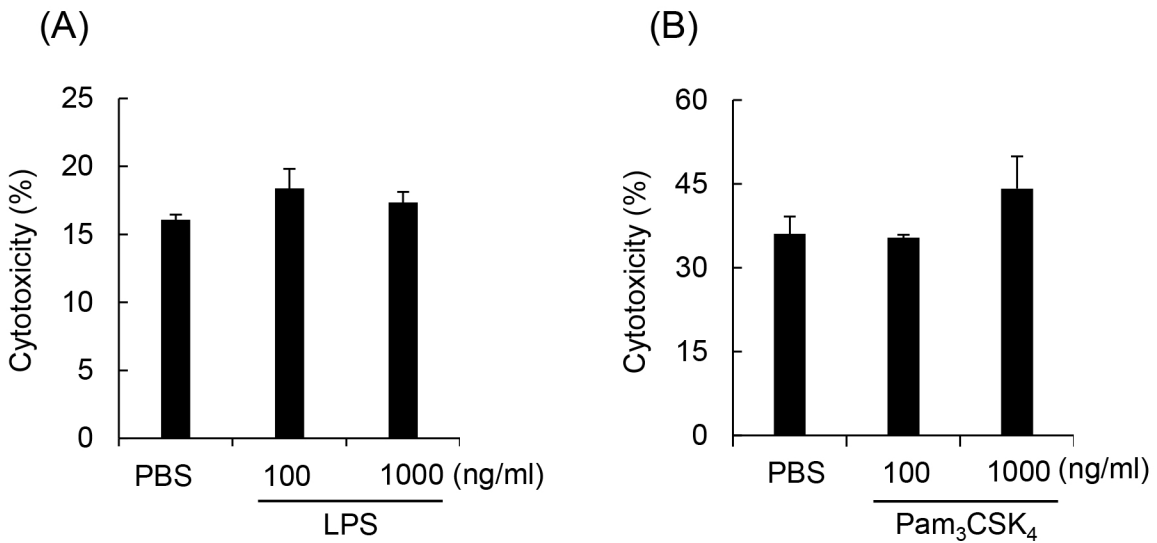

(A)
Cytotoxicity (%)
25
20
15
10
5
0
PBS
100
1000 (ng/ml)
LPS
(B)
Cytotoxicity (%)
60
45
30
15
0
PBS
100
1000 (ng/ml)
Pam3CSK4

Figure 7

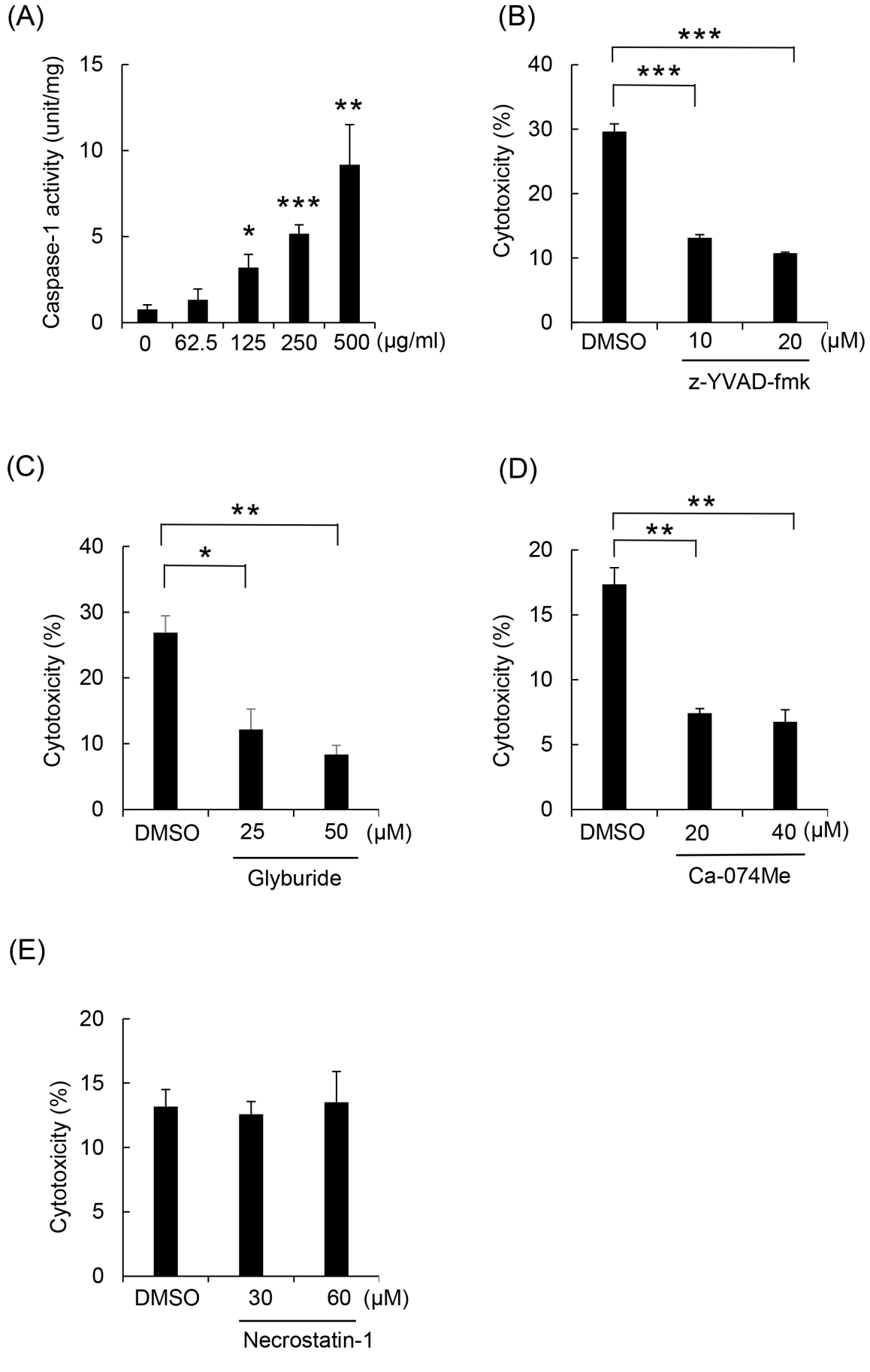
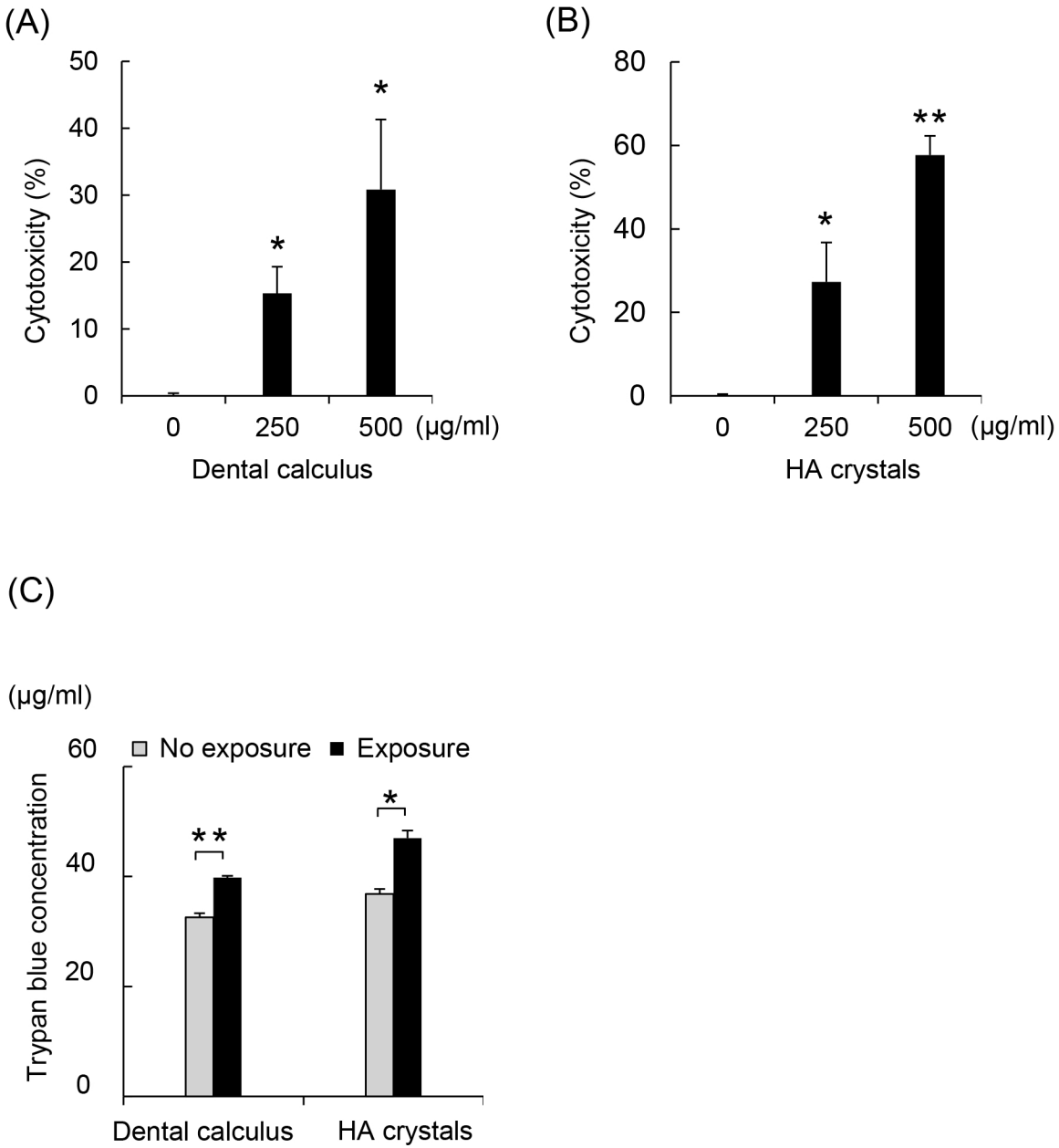

(A)
Caspase-1 activity (unit/mg)
15
10
5
0
*
***
**
0
62.5
125
250
500 (μg/ml)
(B)
***
***
Cytotoxicity (%)
40
30
20
10
0
DMSO
10
20 (μM)
z-YVAD-fmk
(C)
**
*
Cytotoxicity (%)
40
30
20
10
0
DMSO
25
50 (μM)
Glyburide
(D)
**
**
Cytotoxicity (%)
20
15
10
5
0
DMSO
20
40 (μM)
Ca-074Me
(E)
Cytotoxicity (%)
20
15
10
5
0
DMSO
30
60 (μM)
Necrostatin-1

Figure 8


(A)
Cytotoxicity (%)
50
40
30
20
10
0
*
*
0
250
500
(μg/ml)
Dental calculus
(B)
Cytotoxicity (%)
80
60
40
20
0
*
**
0
250
500
(μg/ml)
HA crystals
(C)
(μg/ml)
No exposure
Exposure
Trypan blue concentration
60
40
20
0
**
*
Dental calculus
HA crystals