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Butyrate stimulates Histone H3 acetylation, RANKL expression, but inhibited osteoprotegerin expression/secretion in MG-63 Osteoblastic Cells

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Running title: Toxicity of butyrate on osteoblasts

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Abstract

Butyric acid as a histone deacetylase (HDAC) inhibitor was produced by a number of periodontal and root canal microorganisms (such as *Porphyromonas*, *Fusobacterium* etc.). Butyric acid may affect the biological activities of periodontal/periapical cells such as osteoblasts, periodontal ligament cells etc., and thus affect periodontal/periapical tissue destruction and healing. The purposes of this study were to study the toxic effects of butyrate on matrix and mineralization markers' expression of MG-63 osteoblasts. Cell viability and proliferation were determined by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. Cellular apoptosis and necrosis were analyzed by propidium iodide/Annexin V flow cytometry. Protein and mRNA expression of OPG, and RANKL were analyzed by western blotting and RT-PCR. OPG, soluble RANKL (sRANKL), 8-isoprostane, pro-collagen I, MMP-2, osteonectin (SPARC), osteocalcin and osteopontin secretion into culture medium were measured by enzyme-linked immunosorbant assay. Histone H3 acetylation levels were evaluated by immunofluorescent staining (IF) and western blot. We found that butyrate induced morphologic changes of growing MG-63 cells, with bigger and flattened in appearance. Butyrate activated histone H3 acetylation of MG-63 cells. Exposure of MG-63 cells to butyrate partly decreased cell number with no marked increase in apoptosis and necrosis. Butyrate stimulated RANKL protein expression, whereas it inhibited OPG protein expression. Butyrate also inhibited the secretion of OPG in MG-63 cells, whereas sRANKL level was below detection limit. Butyrate stimulated 8-isoprostane, MMP-2 and osteopontin secretion, but not procollagen I, osteonectin, osteocalcin in MG-63 cells. In conclusion, butyric acid generated by periodontal and root canal microorganisms may potentially induce bony destruction and impair bone repair by alteration of OPG/RANKL expression/secretion, 8-isoprostane, MMP-2, and osteopontin secretion, and affect cell proliferation. These effects are possibly related to increased histone

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acetylation. These events are important in the pathogenesis of periodontal and periapical destruction.

Keywords: Bone resorption; Butyric acid; HDAC inhibitor; Osteoblasts; Osteoprotegerin/RANKL; Periodontal/root canal pathogens

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1. Introduction

Butyrate is a well-known histone deacetylase (HDAC) inhibitor [1]. Butyric acid can be generated by microorganisms present in the dental plaque and biofilms of the root surface and root canals, and involve in the pathogenesis of periodontitis and apical periodontitis. Periodontal and root canal (tooth pulp) pathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*) or other microorganisms, are widely distributed in the periodontal pocket, root canal and the apical area of the patients [2-7]. These pathogenic microorganisms are capable of attack, invading tissue cells or releasing toxic metabolites such as endotoxin, proteases, short chain fatty acids (e.g., propionic acid, butyric acid), which affect cellular responses [6-8].

Recent studies have found that butyrate plays an important role in periodontal and apical lesions in the gums and periodontal tissues. The concentrations of butyric acid may reach 0.2-16 mM in gingival sulcus [9,10] and the concentration of butyric acid in the root canals decreased after endodontic treatment [11]. Butyric acid may affect biological activities of periodontal/periapical cells such as osteoblasts, periodontal ligament cells, and gingival fibroblasts (GF) and inflammatory cells etc. [12-14], and thus affect periodontal/periapical tissue destruction and healing.

Bone homeostasis depends on the balance of bone resorption by osteoclasts and formation by osteoblasts. An imbalance of bone turnover may cause diseases such as osteoporosis, bone resorption, periodontitis etc. [15]. Interaction between osteoblasts and osteoclasts via direct contact or cytokine release is important for bone homeostasis. Osteoblasts may affect osteoclast activity through OPG/RANK/RANKL system [15]. An increase in RANK, RANKL and OPG expression in the progression of periodontitis and apical periodontitis has been reported [16-18]. An increase ratio of RANKL/OPG can be a marker showing the presence of periodontitis and bone resorption [18]. These changes in expression of OPG and RANKL during periodontitis may be related to the presence of various periodontal

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and root canal pathogens [19,20] and related toxins. *P. gingivalis* LPS has been shown to affect RANKL and RANKL/OPG ratio of periodontal ligament fibroblasts [21]. However, limited information is known about butyric acid on OPG and RANKL expression of osteoblastic cells and its possible contribution in periodontal bony destruction.

Previously we have found that butyrate suppresses the adhesion, cell growth, protein synthesis, but induce reactive oxygen species and cell cycle arrest of gingival fibroblasts (GF) [22,12]. It also inhibited cell growth, collagen I expression, and induces cell cycle arrest, p21 and p27 expression of osteoblasts [23]. The purposes of this study were to further study the toxic effects (OPG, RANKL) of butyrate on MG-63 osteoblasts and the related mechanisms. Understanding these effects and mechanisms can help us with the treatment and prevention of subsequent periodontal and periapical diseases

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2. Results

2.1. Stimulation of Histone H3 Acetylation by Butyrate

Control MG63 cells showed limited nuclear staining of Ac-H3 **(Figure 1A)**. Butyrate (8 mM) stimulated the histone H3 acetylation of MG-63 cells as analyzed by IF. An increase in red fluorescence of nuclear staining of MG-63 cells was noted after 120 min of exposure o 8 mM butyrate **(Figure 1A).** An increase in Ac-H3 nuclear staining was also noted when MG-63 cells were exposed to butyrate for 24 hours **(Figure 1B).** Accordingly, butyrate stimulated Ac-H3 expression of MG-63 cells as analyzed by western blotting **(Figure 1C).**

2.2. Morphology of MG-63 Cells after Exposure to Butyrate for 3 Days

When non-confluent MG-63 cells (1 x 10^4 cells/well) were cultured for 3 days, cells grew to confluence. MG-63 cells were fibroblast-like in appearance **(Figure 2A)**. When exposed to butyrate (4 mM and 8 mM) for 3 days, cell density of MG-63 cells decreased **(Figure 2B, 2C)**. Exposure to 16 mM for 3 days further decreased the cell density with space between cells. Cell became bigger and flattened in appearance **(Figure 2D)**.

2.3. Effect of Butyrate on the Growth and Cytotoxicity of MG-63 Cells

Accordingly, when non-confluent MG-63 cells (1 x 10^4 cells/well) were exposed to butyrate (16 and 24 mM) for 3 days, viable cell number decreased **(Figure 3A)**. On the other hand, when confluent MG-63 cells (1 x 10^5 cells/well) were exposed to butyrate for 3 days, viable cell number showed no marked difference **(Figure 3B)**.

2.4. Effect of Butyrate on Apoptosis and Necrosis of MG-63 Cells

PI and Annexin V flow cytometric analysis was used to determine the induction of apoptosis and necrosis of MG-63 cells after exposure to various concentrations of

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butyrate. As shown in **Figure 4A,** exposure to 16 mM butyrate could not evidently induce the apoptosis (UR & LR) and necrosis (UL) of MG-63 cells. Quantitatively, the percentage of cells (%) residing in the UL (necrotic cells) increased from 4.19% to 4.79% after exposure to 24 mM butyrate. In addition, the percentage of cells in UR (apoptotic cells), and LR (pro-apoptotic cells) quadrant changed from 0.85% and 0.41% in control to 1.28% and 1.05% respectively by 16 mM butyrate **(Figure 4B, Table 1)**.

2.5. Effect of Butyrate on OPG and RANKL mRNA and Protein Expression

Butyrate stimulated RANKL mRNA expression (> 1 mM), whereas it inhibited OPG mRNA expression as analyzed by RT-PCR **(Figure 5A**), and thus decreased the ratio of OPG/RANKL. Butyrate also stimulated RANKL protein expression (> 4 mM). On the contrary it inhibited OPG protein expression (> 1 mM) as analyzed by western blotting **(Figure 5B**).

2.6. Effect of Butyrate on OPG and RANKL Secretion of MG-63 Cells

Since OPG and RANKL secretion is important for bone turnover, we further determined their secretion MG-63 cells. Interestingly butyrate markedly inhibited the secretion of OPG in MG-63 cells at concentrations ranging 2-16 mM **(Figure 5C).** Unexpectedly sRANKL level in the culture medium was below detection limit **(data not shown)**.

2.7. Effect of Butyrate on 8-Isoprostane, Pro-collagen I, MMP-2, Osteopontin, Osteonectin, and Osteocalcin Secretion of MG-63 Cells

Exposure to butyrate stimulated 8-isoprostane production of MG63 cells **(Figure 6A)**. For matrix turnover, butyrate showed no marked effect on pro-collagen 1a1 secretion after 24 hours of exposure **(Figure 6B**). On the other hand, butyrate (> 1

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mM) induced MMP-2 secretion of MG-63 cells **(Figure 6C)**. For mineralization markers, butyrate slightly decreased the osteonectin (SPARC) secretion, slightly increased the osteocalcin production of MG-63 cells ($p>0.05$) **(Figure 6D, 6E)**. Moreover, butyrate stimulated osteopontin secretion of MG63 cells **(Figure 6F)**.

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3. Discussion

Butyrate is a well-known histone deacetylase (HDAC) inhibitor produced by microorganisms of the oral cavity and intestinal tract. Microorganisms in the root canals and periodontal pocket may generate short chain fatty acids (SCFA) including propionic acid and butyric acid, relating to periodontal/periapical tissue toxicity [3,14]. The levels of SCFA may reach 0.2-16 mM in gingival crevicular fluid, and periodontal treatment may decrease the concentrations of SCFA in GCF [9,10,14]. In addition to P. gingivalis, other root canal microorganisms such as *Fusobacterium, Actinobacteria phylum, Parvimonas micra* may also generate SCFA and treatment may decrease SCFA levels [11]. They may also release various toxic factors (fimbria, endotoxin, short chain fatty acids etc.) to induce inflammatory response, and affect the viability and biological activities of periodontal and periapical tissues, leading to bony destruction. In this study, butyrate inhibited the proliferation, but showed little cytotoxic effect to MG-63 cells. Accordingly, butyrate induces minimal apoptotic and necrotic effect on MG-63 cells as analyzed by PI and annexin V flow cytometry. Other studies have reported the apoptotic effect of butyrate on colon cancer cells, B cells and cells [1,24,25]. Similarly butyrate suppressed the adhesion, growth and protein synthesis of gingival epithelial cells and fibroblasts. It also induced the death and cytokine release of inflammatory cells [9,12,14,22]. Ho & Chang (2007) also found the toxicity of butyrate to dental pulp cells and its relation to glutathione (GSH) depletion [26]. Interestingly proliferating MG-63 cells became bigger and flattened in appearance after exposure to butyrate. While mesenchymal stem cells are found to become flatten and spread to differentiate into osteoblasts [27], and butyrate is shown to affect differentiation of colon cancer cells and osteoblastic cells [1,24,28,29]. Butyrate (0.1 mM) stimulates the expression of mineralization markers in periodontal ligament cells, whereas butyrate exhibits cytotoxicity at

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concentrations higher than 1 mM [30]. The actual reasons and meanings for different results by butyrate are unclear and await further investigation.

SCFA have inflammatory and anti-inflammatory effects depending on cells and conditions [31,32]. As a HDAC inhibitor, butyrate may stimulate histone acetylation [1]. Interestingly, butyrate is shown to attenuate the TNF-α and LPS-induced IL-6, but not IL-8 of endothelial cells, via GPR41/GPR43 receptors. Butyrate prevents the TNF-α and LPS-induced IL-8 expression is related to inhibition of histone deacetylase (HDAC) [33]. Butyrate stimulates differentiation of mesenchymal stem cells to smooth muscle cells or osteoblasts by enhance histone H3 and H4 acetylation, down-regulation of HDAC2 or MEK/ERK-Runx2 signaling [34,35]. In this study, butyrate also induced the histone H3 acetylation of MG-63 osteoblasts. Recently stimulation of histone acetylation by HDAC inhibitors such as trichostatin A, butyrate, and valproic acid, has been shown to promote osteoblast maturation and bone formation [36]. During osteoblast differentiation, histone H3 and H4 are hyperacetylated. Knockdown of HDAC1 by siRNA also promote osteoblast differentiation [37]. These results suggest the possible effect of butyrate on bone turnover.

Previous study has found the stimulation of reactive oxygen species (ROS) production of MG-63 and other cells [23]. 8-Isoprostane (8-iso-prostaglandin F2α), as an oxidative stress marker and lipid peroxidation product, is generated by free-radical catalyzed peroxidation of essential fatty acid such as arachidonic acid, to stimulate redox-sensitive signaling pathways and transcriptional factors [38-40]. Isoprostanes may mediate vasoconstriction, tissue inflammation, perception of pain, vascular reperfusion, paracetamol poisoning, liver cirrhosis, atherosclerosis and cancer [38,39,41]. Limited information is known about the generation of isoprostanes by osteoblasts and their roles in bone turnover. In this study, 8-isoprostane as an oxidative stress marker was also found to be stimulated in

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MG-63 cells by butyrate, suggesting the induction of lipid peroxidation by butyrate. This may partly explain the elevated salivary, gingival crevicular fluid or root canal 8-isoprostane level in patients with periodontitis, pulpitis or chronic apical periodontitis [42-45], suggesting their involvement of increased oxidative stress in pulpal/periapical pain, periodontal and periapical bony destruction. It has been found that iso-PGE_2 but not iso-PGF_2 alpha had an inhibitory effect on the induction of alkaline phosphatase activity in MC3T3-E1 preosteoblasts [46]. Urinary F2-isoprostanes levels are found to show negatively correlation with bone mineral content and bone mineral density [47]. It has been found that patients with diabetes, obesity, hypercholesterolemia, smokers etc. have higher levels of urinary isoprostane [48]. Possibly, urinary isoprostane levels can be used as marker of periodontal and periapical diseases in the future. Increase of 8-isoprostane by butyrate suggests its involvement in the pathogenesis of periodontal/periapical diseases.

Bone turnover is tightly regulated by interaction of osteoblasts and osteoclasts [15], possibly via OPG/RANKL system. It has been shown that periodontal/root canal pathogens may stimulate RANKL expression of bone marrow cells [19,20]. Injection of anti-RANKL and osteoprotegerin fusion protein into rat gingiva may attenuate bone resorption induced by *P. gingivalis* infection [20]. *P. gingivalis* LPS is shown to induce RANKL and RANKL/OPG ratio in periodontal ligament fibroblasts [21]. An increased expression of RANK, RANKL and OPG during the progression of periodontitis and apical periodontitis has been reported [16]. Belibasakis et al. (2013) also found the protective role of OPG against root resorption and the increased apical RANKL/OPG ratio in bone resorption [17]. Level of RANKL is found to be increased, but OPG level is decreased in severe periodontitis [49]. Cyst and granuloma tissues from periapical lesions also show the increased expression of RANKL, and important of

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RANKL/OPG in apical bony destruction [17,50-52]. However, butyrate is shown to stimulate OPG expression in normal human osteoblasts [29]. In this study, butyrate was shown to stimulate RANKL, but decrease OPG expression and secretion of osteoblasts. This may partly explain why butyric acid may be involved in periodontal and periapical bony destruction and turnover.

Butyrate (0.1 mM) stimulates calcium content, mineralized nodule formation and the expression of bone sialoprotein (BSP), osteopontin (OPN) and OPG, but showed little effect on proliferation, M-CSF, type I collagen expression and ALP activity of normal human osteoblasts [29]. Butyrate affects mainly pre-osteoblasts but not mature osteoblasts. It also stimulates ALP activity in MC3T3 osteoblasts [28]. Butyrate stimulates BSP expression in rat ROS17⁄2.8 osteoblast-like cells [1]. Bone sialoprotein (BSP) may function in the initial mineralization stage of bone, and is crucial for osteoblast differentiation, bone matrix mineralization and tumor metastasis [1]. On the contrary, butyrate (1 mM) suppressed the expression of Runx2, Osterix, Dlx5, Msx2, osteocalcin, ALP and BSP expression as well as mineralized nodules formation in ROS17⁄2.8 osteoblasts [13]. Similarly butyrate (0.1-1 mM) also stimulate COX-2, collagen, OPN, EP1, and EP2 receptors' expression, and PGE2 production, but showed little effect on proliferation of ROS17⁄2.8 cells [53]. In this study, butyrate stimulated OPN, and MMP-2, but showed no marked effect on pro-collagen I, osteonectin (SPARC) and osteocalcin of MG63 osteoblastic cells. These results suggest that butyrate may affect matrix turnover and mineralization. The differential effects of butyrate may be related to differentiation status (pre-osteoblasts and osteoblasts), butyrate concentration, exposure time, cell density, and cell type etc.

We have previously found that propionate and butyrate may affect the activities including growth, attachment, migration, reactive oxygen species and cell cycle alteration of gingival fibroblasts and osteoblasts [12,22,23]. In conclusion,

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exposure of MG-63 osteoblastic cells to butyrate leads to histone H3 acetylation and 8-isoprostance production. Butyrate inhibited the proliferation, but not cytotoxicity and apoptosis of MG-63 osteoblasts. It further stimulates MMP-2, OPN, RANKL, but inhibit OPG expression and secretion, affecting matrix turnover and mineralization of bone tissues. These results may facilitate our understanding the role of butyric acid in the pathogenesis of apical and periodontal bony destruction, and can be helpful for disease prevention and treatment in the future.

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4. Materials and methods

4.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin/EDTA, penicillin/streptomycin were from Gibco (Life Technologies, Grand Island, NY, USA). Antibodies of glyceroaldehyde-3-dehydrogenase (GAPDH) were purchased from Santa Cruz, whereas antibodies for Ac-H3, OPG and RANKL were from GeneTex. Ethidium bromide, agarose and kits for reverse transcription (RT) and polymerase chain reaction (PCR) are purchased from HT Inc., UK. Total RNA isolation kits are from Qiagen Inc. (Santa Clarita, CA, USA). Specific PCR primer sets will be synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA, USA). Protein assay kits were obtained from Bio-Rad (Bio-Rad Labs, Hercules, CA, USA). Sodium butyrate was obtained from sigma (Sigma-Aldrich Company, St. Louis, MO, USA). MG-63 cells were obtained from American Type Culture Collection (ATCC). OPG, RANKL, pro-collagen I, MMP-2, osteopontin, osteocalcin and osteonectin (SPARC) ELISA kits were from R&D. 8-Isoprostane ELISA kits were from Cayman.

4.2. Immunofluorescent Staining of Ac-H3 Expression

MG-63 cells (1×10^5) were seeded onto 24-well culture with sterile coverslips in 1 ml DMEM with 10% FBS. After 24 hr, culture medium containing various concentrations of butyrate (1-16 mM) was added and cells were further incubated for 24 h. MG-63 cells were also exposed to 8 mM butyrate for different time points (5-120 min). Medium was removed, and cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min. Cells were further washed with PBS, permeabilized with 2% Triton X-100, exposed to 0.3% v/v H_2O_2 for 20 minutes. After rinse with PBS, 5% bovine serum albumin (BSA) was used for blocking cells for 1 h and then cells were incubated with primary antibodies (Acetyl-Histone H3) (1:1000, v/v) at room

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temperature for overnight. Following washed by PBS, cells were incubated in TRITC-conjugated secondary antibodies in the dark for 1 h and counterstained the nucleus with DAPI (1:1000) for 30 min. Finally the cells in coverslips were mounted and photographed/observed under an inverted microscope and DP Controller/Manager software (Olympus IX71, Olympus Corporation) [23,54].

4.3. Effect of Butyrate on Proliferation and Viability of MG-63 Cells

Briefly 1 x 10^4 and 1 x 10^5 MG-63 cells/24-wells were exposed to fresh medium containing various concentrations butyrate (2, 4, 8, 16 and 24 mM) for 24 hours or 3 days. Morphology of cells was taken under microscope. Viable cell number of MG-63 cells was estimated by MTT assay [22,23]. Briefly, culture medium was collected and then fresh medium containing MTT (0.5 mg/ml) was added to the attached MG-63 cells. After 2-hours of incubation, medium with MTT are removed, and DMSO was added to the wells to dissolve formazan. The viability of the cells in each well was measured by determination of optical density at 540 nm by a Microplate Reader.

4.4. Induce the Apoptosis and Necrosis of MG-63 Cells by PI and Annexin V Flow Cytometric Analysis

For annexin V/PI dual staining flow cytometry [55,56], 5 x 10^5 MG-63 cells/6-wells were exposed to 2 ml fresh medium containing different concentrations of butyrate (2, 4, 8, 16 and 24 mM) for 24 hours. Cells were collected and re-suspended in 400 µl, 10 mM HEPES solution (pH 7.4) containing 140 mM NaCl and 2.5 mM CaCl2. Then 10 µl of PI (50 µg/ml) and 5 µl of Annexin V-FITC were added into cells for further incubation in the dark for 30 min. After washing, the stained cells were subjected immediately to FACS Calibur Flow Cytometry (Becton Dickinson, USA) analysis of annexin V-FITC and PI fluorescence. The fluorescence of cells of samples was gated and counted. The percentage of cells localization in the upper right (apoptotic),

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upper left (necrotic cells), lower right (pro-apoptotic cells), and lower left (control) portion of the histogram was determined for comparison.

4.5. Effect of Butyrate on the Expression of OPG, RANKL of Cultured MG-63 Cells

About 1 x 10^6 MG-63 cells in 10-cm culture dishes were exposed in fresh medium containing butyrate (1, 2, 4, 8, 16 mM). Total RNA was isolated using Qiagen RNA isolation kits. ***Semi-quantitative Reverse-transcriptase and Polymerase Chain Reaction (RT-PCR)-*** In brief, 3 µg of denatured total RNA was reverse transcripted in a total volume of 44.5 µl reaction mixture containing 4 µl of random primer (500 µg/ml), 8 µl of dNTP (2.5 mM), 4.5 µl of 10x RT buffer, 1 µl of RNase inhibitor (40 U/µl) and 0.5 µl of RT (21 U/µl) at 42^{O}C for 90 minutes. Four microliters of cDNA were then used for PCR amplification in a reaction volume of 50 µl containing 5 µl of 10x Super TAQ buffer, 4 µl of dNTP (2.5 mM), 1 µl of each specific primer, and 0.2 µl of Super TAQ enzyme (2 U/µl). The reaction mixture was initially heated to 94^{O}C for 5 minutes in the first cycle, then the reaction was amplified for 15-35 cycles of 94^{O}C for 30 sec, 55^{O}C for 30 sec and then 72^{O}C for 30 sec with a thermal cycler (Perkin Elmer 4800, PE Applied Biosystems, Foster city, CA, USA). Finally, the reaction was set at 72^{O}C for further 10 minutes.

Specific primer sets for OPG: TCAAGCAGGAGTGCAATCG and AGAATGCCTCCTCACACAGG (342 bp); RANKL: CCAGCATCAAAATCCCAAGT and CCCCTTCAGATGATCCTTC (603 bp) [57]. Expression of beta-actin was used as control [58]. The amplified DNA products are loaded onto a 1.8% of agarose gel in 1x of TBE buffer for electrophoresis. Gels are stained with ethidium bromide and photographs are taken. The range of amplified DNA product that is linear in relation to the input RNA will be used for data presentation. Amplification of the BAC gene is used for a control.

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4.6. Effects of Butyrate on the OPG, RANKL and Ac-H3 Expression of MG-63 Cells

MG-63 cells (1x 10^6 cells) in 10 cm culture dishes were exposed to fresh medium containing various concentrations of butyrate (1, 2, 4, 8, 16 mM) for 24 hrs. Cell lysates were collected as described previously using freshly prepared lysis buffer (10 mM Tris-HCl, pH 7; 140 mM sodium chloride; 3 mM magnesium chloride; 0.5% NP-40; 2 mM phenylmethylsulfonyl fluoride; 1% aprotinin; and 5 mM dithiothreitol) [12,58-60]. The protein concentration of the cell lysates are measured by Bio-Rad protein assay kits. Equal amounts of protein (50 µg/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (Scie-Plas, UK) and transferred to PVDF membrane by electroblotting. The membrane was blocked for 30 min at room temperature in a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.1% sodium azide) and then incubated for 2 hr with anti-human OPG, RANKL, Ac-H3 (1:500) and GAPDH antibodies. Membranes were washed three times with TBST (10 mM Tris, pH 7.5; 100 mM NaCl, 0.1% Tween-20) for 10 min each, and then incubated with HRP-labeled goat anti-mouse secondary antibody for 1 hr. The membrane was then washed 4 times with TBST. Finally the immunoreactive bands were developed by Enhanced Chemiluminescence (ECL) reagent and visualized on Fuji X-ray film.

4.7. Effect of Butyrate on OPG, RANKL, 8-Isoprostane, Pro-collagen I, MMP-2, Osteopontin, Osteonectin and Osteocalcin Secretion of MG-63 Cells

Cells were treated by butyrate as above (Section 1.2). Culture medium was collected before MTT assay and used for enzyme-linked immunosorbant assay (ELISA) of OPG, RANKL, procollagen I, MMP-2, osteopontin, osteonectin, osteocalcin and 8-isoprostane levels according to the instruction of ELISA kit.

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4.8. Statistical Analysis

Three or more independent experiments were performed. The results were statistically analyzed by paired Students *t*-test. A p value < 0.05 was regarded to have a statistically significant difference between groups.

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Table 1. Induction of apoptosis and necrosis of MG63 cells by various concentrations of butyrate as analyzed by PI and Annexin V flow cytometry (n=4)

	Control	8 mM Butyrate	16 mM Butyrate	24 mM Butyrate
UR	0.85 ± 0.10	1.25 ± 0.19	1.28 ± 0.2	1.43 ± 0.40
UL	4.19 ± 1.12	4.19 ± 0.57	4.24 ± 0.51	4.79 ± 0.33
LR	0.41 ± 0.06	0.86 ± 0.21	1.05 ± 0.30	0.75 ± 0.24
LL	94.54 ± 1.05	93.69 ± 0.31	93.41 ± 0.18	93.02 ± 0.36

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Figure Legends

Figure 1. Stimulation of Histone H3 acetylation of MG63 cells as analyzed by IF and western blotting. **(A)** IF pictures of Ac-H3 expression: control (0 min), and Butyrate (8 mM)-treated MG63 cells for 120 min. **(B)** IF pictures of Ac-H3 expression: Control (24 hours) and Butyrate (8 mM)-treated MG63 cells for 24 hours, **(C)** Western blotting of control and 8 mM butyrate-treated MG-63 cells for 24 hours. One representative IF study result was shown.

Figure 2. Morphologic changes of MG63 cells after exposure to butyrate for 3 days. MG63 cells (1 x 10000 cells/24-well) were exposed to butyrate for 3 days. **(A)** Control MG 63 cells, **(B)** Exposure to 4 mM butyrate for 3 days, **(C)** Exposure to 8 mM butyrate for 3 days, **(D)** Exposure to 16 mM butyrate for 3 days. 100x original magnification. One representative result was shown.

Figure 3. Effect of butyrate on the growth and cytotoxicity of MG63 cells **(A)** MG63 cells (1 x 10000 cells/24-well) were exposed to butyrate for 3 days, **(B)** About confluent MG63 cells (1 x 100000 cells/24-well) were exposed to butyrate for 3 days. Cell number was estimated by MTT assay. Results were expressed as percentage of control (Mean ± SE). *denotes statistically significant difference when compared with control ($p<0.05$).

Figure 4. Effect of butyrate in the induction of apoptosis and necrosis of MG63 cells as, analyzed by PI + Annexin V flow cytometry. UL: necrosis, UR and LR: apoptosis, One representative PI and Annexin V flow cytometry histogram was shown. **(A)** Control, and 16 mM butyrate-treated cells, **(B)** quantitative analysis of cells residing in upper left, upper right, lower left and lower right region. (Mean ± SE) (n=4)

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Figure 5. Effect of butyrate on OPG and RANKL expression of MG63 osteoblastic cells. (A) RT-PCR analysis of mRNA expression, (B) Western blot analysis of OPG and RANKL protein expression, (C) ELISA analysis of OPG level in culture medium. *denotes statistically significant difference when compared with control ($p<0.05$).

Figure 6. Effect of butyrate on 8-isoprostane, pro-collagen I, MMP-2, osteonectin (SPARC), osteocalcin, and osteopontin (OPN) secretion of MG-63 cells as analyzed by ELISA. (A) 8-Isoprostane level, (B) Pro-collagen I level, (C) MMP-2 secretion, (D) Osteonectin (SPARC) secretion, (E) osteocalcin level, (F) osteopontin (OPN) secretion. Results were expressed as Mean ± SE. *denotes statistically significant difference when compared with control.

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Figure 1

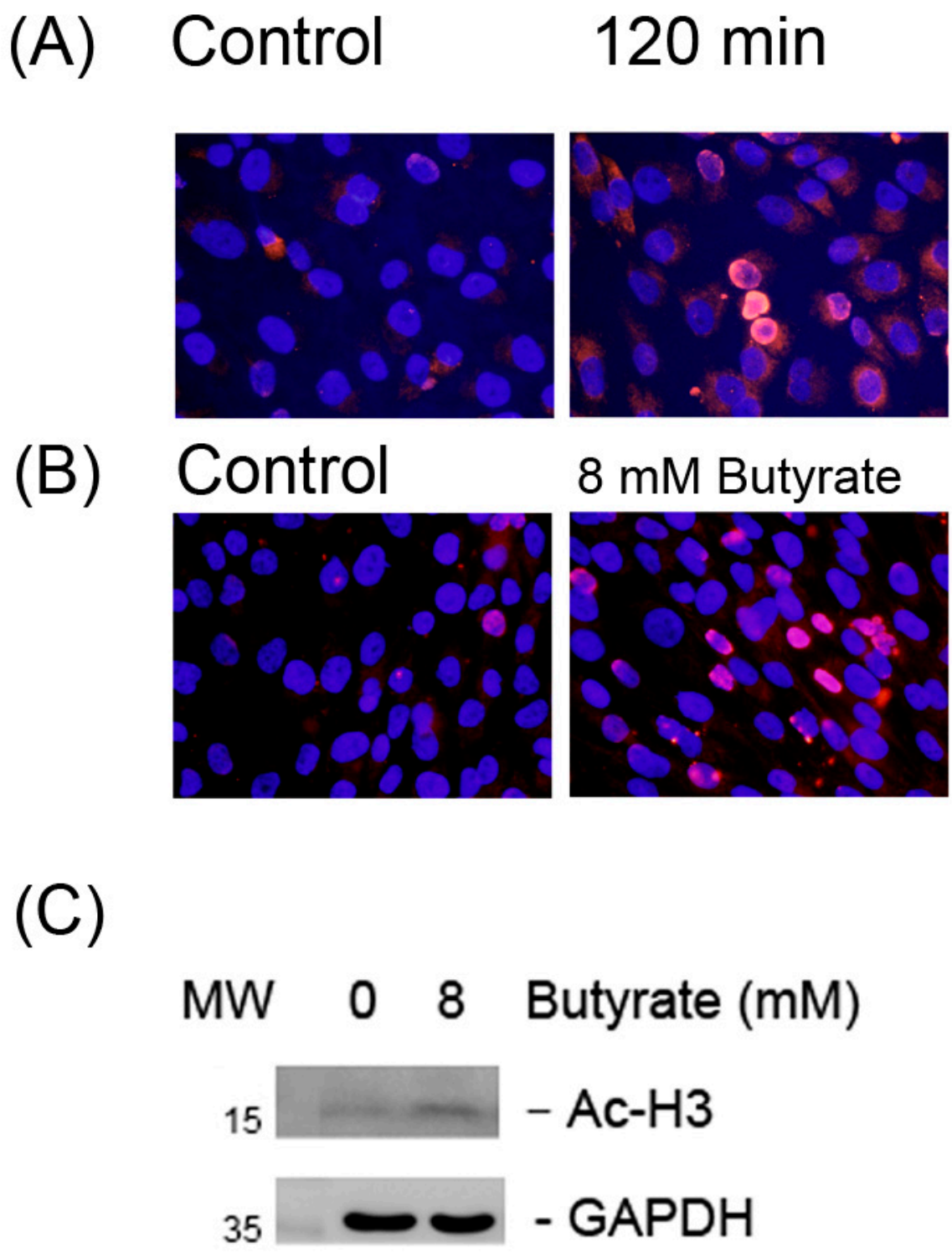

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Figure 2.

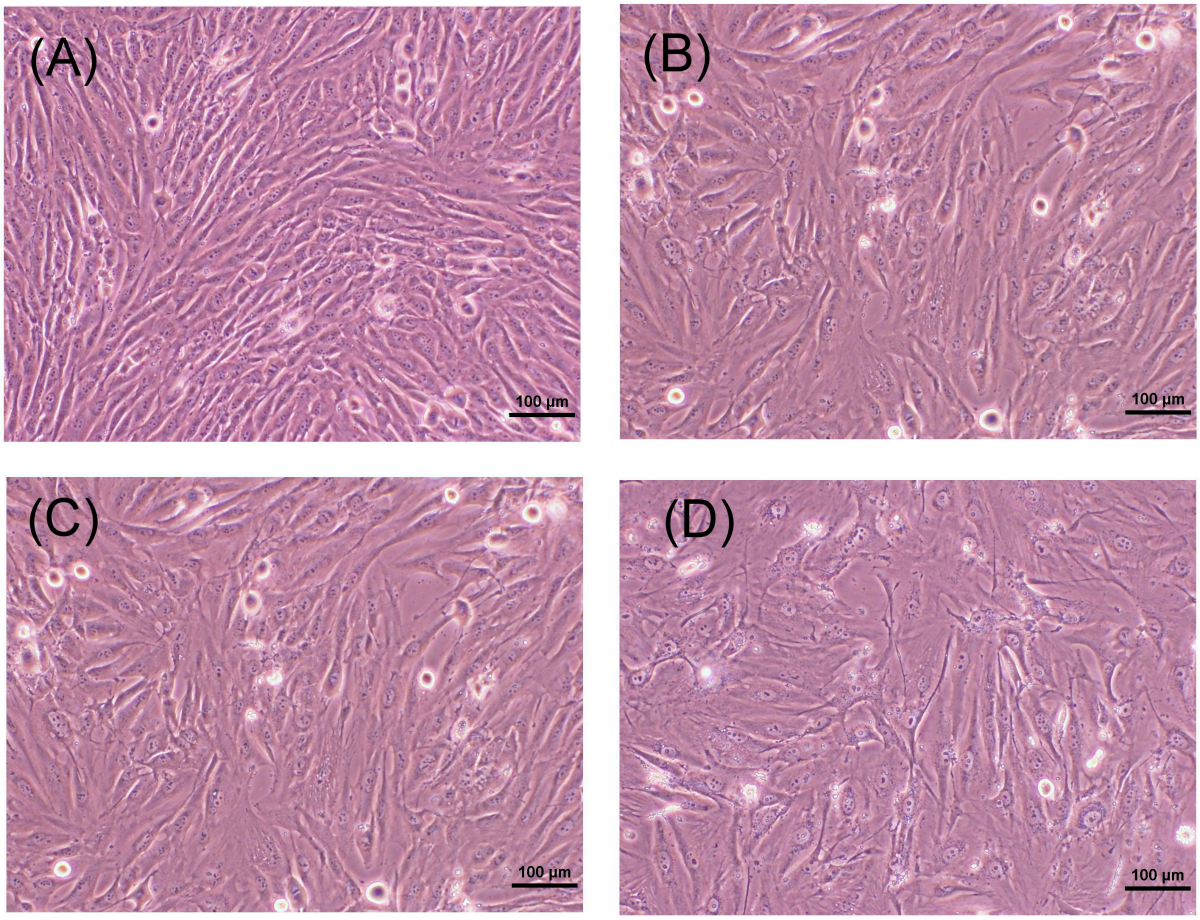

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Figure 3.

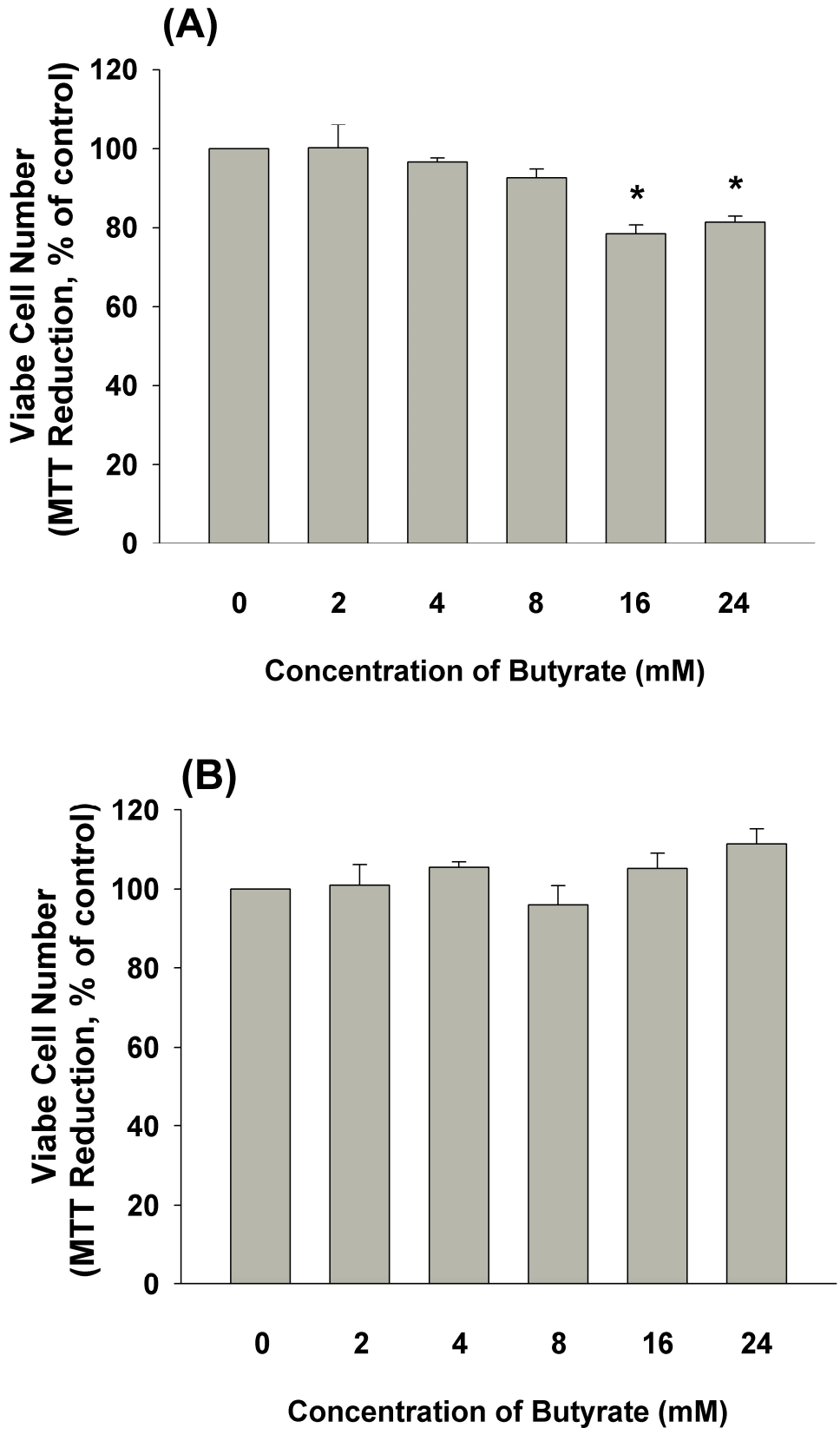

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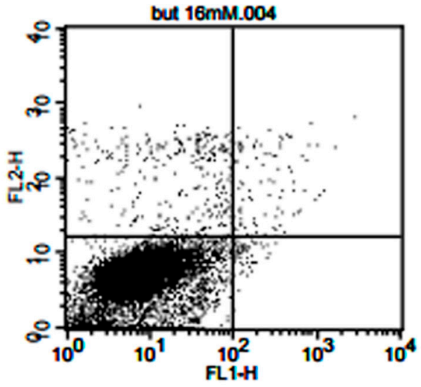
Figure 4

(A) control



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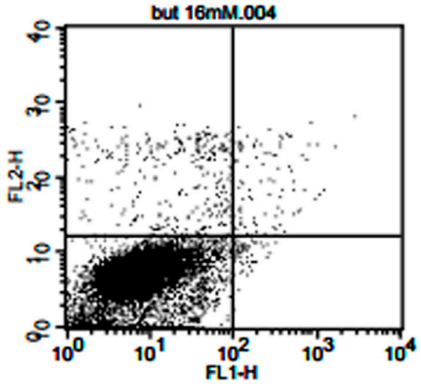
Quad	% Gated	% Total
UL	4.62	4.62
UR	0.92	0.92
LL	93.83	93.83
LR	0.63	0.63

16 mM butyrate



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Quad	% Gated	% Total
UL	4.62	4.62
UR	0.92	0.92
LL	93.83	93.83
LR	0.63	0.63

(B)

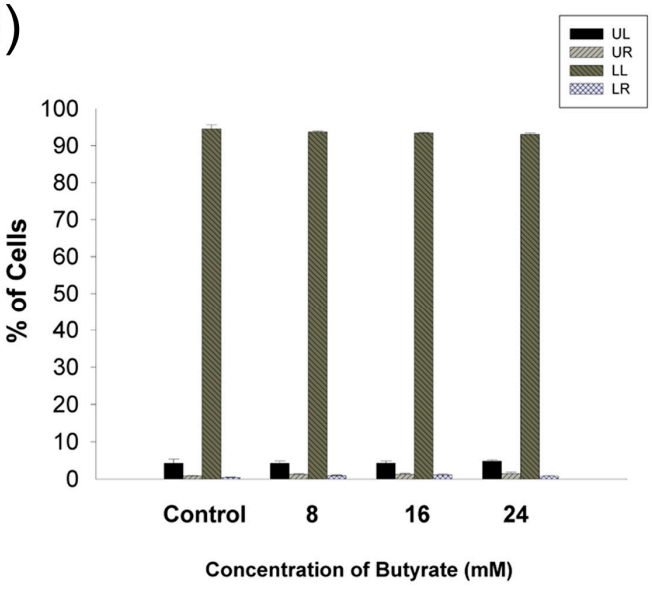

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Figure 5.

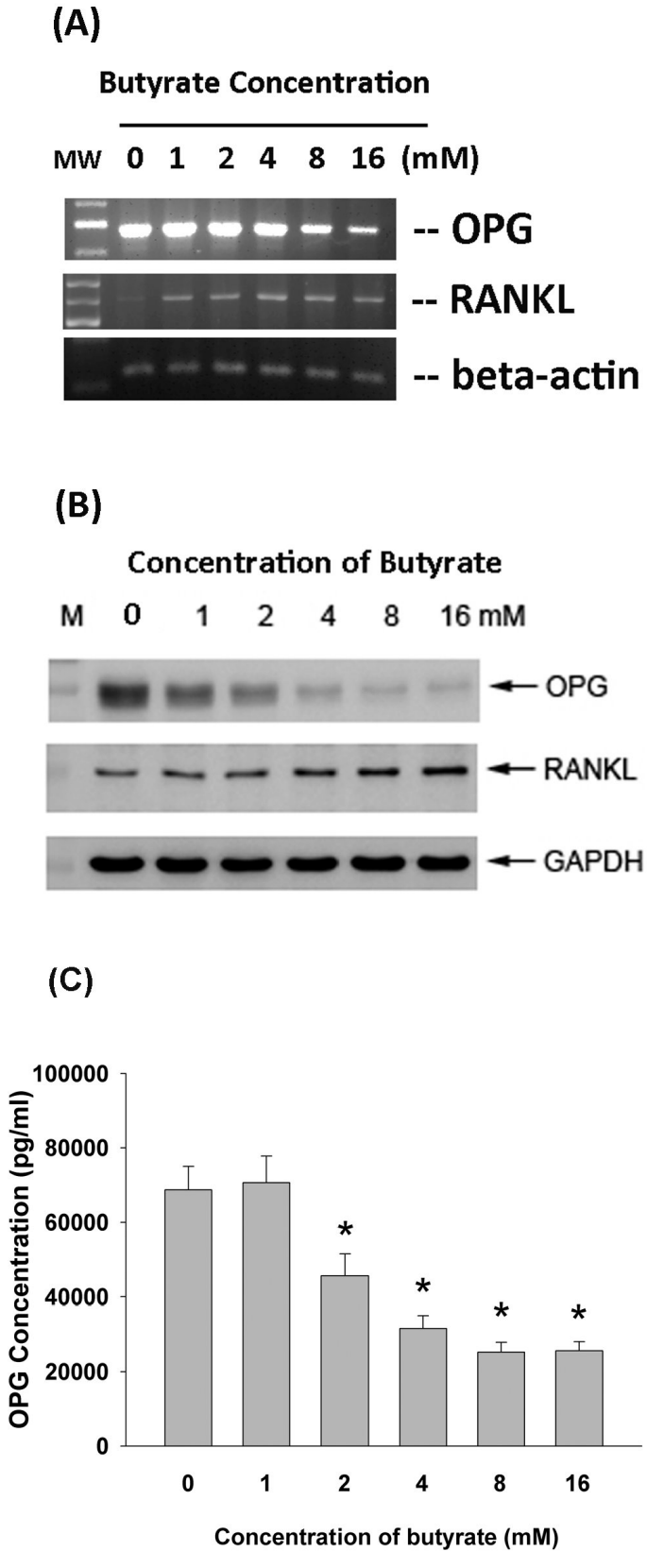

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Figure 6