Calcium-dependent activation of PHEX, MEPE and DMP1 in osteocytes

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Abstract. Calcium (Ca^{2+}) signaling is the first messenger signal exhibited by osteocytes. The present study aimed to better understand the link between Ca^{2+} concentration, and the levels of bone mineralization regulator proteins [phosphate-regulating neutral endopeptidase on chromosome X (PHEX), matrix extracellular phosphoglycoprotein (MEPE) and dentin matrix protein 1 (DMP1)] and the levels of oxidative stress in osteocytes. The viability of MLO-Y4 cells was determined using the live/dead assay following treatment with various Ca^{2+} concentrations (1.8, 6, 12, 18, 24 and 50 mM) for different durations (15 and 60 min, and 24 h). Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and NADPH oxidase (NOX) enzymes were analyzed using a colorimetric method. Apoptosis was detected by caspase-3 analysis. Furthermore, the protein expression levels of PHEX, MEPE and DMP1 were analyzed using immunoblotting, and oxidative stress was examined using the total antioxidant and total oxidant status (TOS) assay. Notably, after 15 min, there were more live cells than dead cells; however, after 60 min, the number of dead cells was increased following treatment with 24 and 50 mM Ca^{2+}. After 24 h, there were more dead cells than live cells following treatment with 50 mM Ca^{2+}. After 24 h of Ca^{2+} treatment, the highest protein expression levels of PHEX, MEPE and DMP1 were measured in cells treated with 24 mM Ca^{2+}. In addition, as Ca^{2+} concentration increased, the TOS and the oxidative stress index values were also increased. In conclusion, these results suggested that 24 mM Ca^{2+} may trigger bone mineralization proteins, such as PHEX, MEPE and DMP1, and could be considered an applicable dosage for the treatment of bone damage in the future.

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Introduction

Osteoblasts and osteoclasts are the major cells of bone that function in bone formation and bone resorption, respectively. Osteocytes are embedded in the mineralized bone matrix and responsible for the regeneration of adult bone cells (1). Osteocytes continue to increase with age and bone size. Because of the limited access to the osteocytes in the bone matrix, knowledge of its functions remained unclear. Due to their particular morphology, they have multiple functions and communication features (2). Kato *et al* developed MLO-Y4 cells, which are remarkably close to primary osteocytes (3) and this cell line, which has high expression of osteocalcin, connexin 43 but low alkaline phosphatase, was obtained from a transgenic mouse. Ca^{2+} and phosphate metabolism is regulated by the osteocyte lacuno-canalicular, which contains multiple proteins such as Phosphate regulating neutral endopeptidase on chromosome X (PHEX), Matrix extracellular phosphoglycoprotein (MEPE), sclerostin, and Dentin matrix protein 1 (DMP1) that can affect systemic mineral homeostasis (4). Osteocytes also play a role in the regulation of calcium and phosphate metabolism which contains multiple proteins such as phosphate regulating neutral endopeptidase on chromosome X (PHEX), matrix extracellular phosphoglycoprotein (MEPE), sclerostin, and dentin matrix protein 1 (DMP1) that can affect systemic mineral homeostasis.

These proteins are secreted by osteocytes and have an essential role in bone modeling and remodeling (5). DMP1 is highly expressed in osteocytes and has a function in bone mineralization. MEPE is a bone mineralization regulator that modifies mineralization within the osteocyte microenvironment in response to mechanical loading. Additionally, MEPE and DMP1 belong to the same SIBLING family (6). Another osteocyte specific protein is PHEX which is related to biomineralization and phosphate homeostasis and sclerostin inhibits osteoblastic bone formation and is expressed and released in osteocytes and other terminally differentiated cell types embedded inside mineralized matrices (7).

Previous studies have shown that these proteins also have a function in the extracellular matrix as a transcription factor (8,9). Ling *et al* showed that bone mineralization was decreased in DMP1 null mice (10). MEPE protein expression

was shown in human bone osteocytes and bone marrow by Rowe *et al* (11). Over-expression of MEPE protein in bone causes a mineralization defect in a murine mice model (12). Lu *et al* reported that MEPE protein was produced by both osteoblasts and osteocytes during skeletogenesis as early two days of the post-natal period (13). It is therefore clear that PHEX, MEPE and DMP1 are secreted by osteocytes. Ca^{2+} is an important intracellular secondary messenger and it is stored in the skeletal system in a form that is linked to carbonated apatite crystals. Ca^{2+}, on the other hand, is found in soluble form in the extracellular domain (14). In osteoblast cells, increased extracellular Ca^{2+} has been shown to trigger intracellular Ca^{2+} signaling (15). In osteoprogenitor cells, increased Ca^{2+} concentration has also been shown to boost proliferation, differentiation, protein matrix production, and mineralized nodule formation in a dose-dependent manner (16). Ca^{2+} and phosphate in the cell culture medium are often analyzed and imaged, especially to quantify mineral deposition rates (17). The measurement of Ca/P ratios used for the identification of the mineralization in the medium can be compared to the ratio in tissue. Similarly, just demonstrating that the culture's Ca^{2+} or phosphate concentration increases over time is insufficient because numerous anionic matrix molecules can bind Ca^{2+}, and the culture's development typically entails changes in matrix protein phosphorylation (18). The cells must produce a collagen matrix on which the mineral will deposit, and that matrix must contain proteins and peptides that support mineralization (19). The addition of serum used in cell culture (which increases Ca^{2+} concentration) provides both mineralization inhibitors and beneficial growth factors (20,21).

There are limited number of studies investigating the Ca^{2+} induced expression levels of bone mineralization proteins in osteocytes. Our study was designed to test the hypothesis, if such biological changes occur as a result of changes in Ca^{2+} concentration, there would be changes in the expression levels of PHEX, MEPE and DMP1 which play an important role in bone formation while osteocytes induced by different Ca^{2+} concentrations. On the other hand, we have shown the apoptosis by evaluating caspase-3 levels and oxidative stress-related enzymes CAT, SOD, GSH, NOX and TAS/TOS levels of MLO-Y4 cell in the presence of different concentrations of Ca^{2+}.

Materials and methods

Cell culture. MLO-Y4 cells were purchased from the Kerafast Company. Cells were grown to confluence in type I rat tail collagen-coated cell culture dishes at 37˚C in a humidified atmosphere containing 5% CO_2 air in 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., 16000044) supplemented α-MEM medium (Gibco; Thermo Fisher Scientific, Inc., 12571063), 5% calf serum (HyClone, SH30401) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc., 15140122).

Live/dead cells assay. The cell viability of MLO-Y4 cell lines was tested using a live/dead assay (Sigma-Aldrich; Merck KGaA, CBA415) at various Ca^{2+} (Sigma-Aldrich; Merck KGaA, 10043-52-4) concentrations (1.8, 6, 12, 18, 24 and 50 mM). After MLO-Y4 osteocytes were seeded $5x10^3$ cells/cm^2 in 48 collagen-coated well plates and incubated for 72 h. The cells were treated with Ca^{2+} at 1.8, 6, 12, 18, 24 and 50 mM concentrations for 15, 60 min and 24 h. After the treatment of MLO-Y4 cells with different Ca^{2+} concentrations for 15 min, 60 min and 24 h, all media were removed and live/dead assay was performed at the end of the time period. The pictures were captured from the same region of interest for each group. At the same time, the percentages of live and dead cells were calculated.

CAT, SOD, GSH and NOX measurements. To detect cellular oxidative stress, the activity of SOD (YLA0115RA), CAT (YLA0123RA), GSH (YLA0121RA) and NOX (YLA1501RA) enzymes were analyzed by using colorimetric diagnostic kits (Shanghai YL Biotech, China). MLO-Y4 cells ($1x10^6$ cells) were seeded in collagen-coated 6 well plates and incubated for 72 h. After the incubation period, the cells were treated with the different concentrations of Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) containing media for 15 and 60 min. High concentrations of calcium led to elevated cell death in 24 h and it was difficult to measure the oxidant-antioxidant enzymes SOD, CAT, GSH, NOX levels in dead cells. So we determined the enzyme levels in 15 and 60th min. At the end of each time point, cells were washed by PBS three times and lysed with the ultrasonic waves to collect. Samples were centrifugated at $2x10^3$ g for 5 min, the supernatants were collected and added to ELISA plate wells with HRP-conjugate reagent and incubated at 37˚C for 60 min. After the incubation, the ELISA plate well was washed five times with the wash buffer. Then, chromogen solution was added to each well and avoided the light for 15 min at 37˚C. After that, stop solution was added to each well. OD value of each well was measured at 450 nm and the activities were calculated according to the standard curve.

Determination of the total antioxidant, oxidant status and oxidative stress index. To determine antioxidant status and oxidant status according to the different concentrations of Ca^{2+} in MLO-Y4 cells, Rel Assay Diagnostic Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) kits (Rel Assay Diagnostic) were utilized. Erel's method was used for the calculations (22). Oxidative Stress Index (OSI) was measured as: OSI=[(TOS, μmol H_2O_2 equivalent/l)/(TAS, mmol Trolox equivalent/l) x10].

Immunoblotting for PHEX, MEPE, DMP1 and GAPDH. Cell lysates were prepared in ice-cold RIPA buffer (Cell Signaling Technology, Inc.) after MLO-Y4 cell lines were treated with six different Ca^{2+} concentrations for 24 h. Cell lysates were spun to remove cellular debris by centrifuge at $12x10^3$ g for 10 min at +4˚C. Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. The gels were transferred on PVDF membrane by using a wet transfer system overnight and were labeled to perform immunoblot analysis with PHEX (Thermo Fisher Scientific, Inc., bs-12313R), MEPE (bioss, bs-8689R), DMP1 (biorbyt, orb255063), GAPDH (Cell Signaling Technology, Inc.) and Horseradish peroxidase-labeled antirabbit secondary antibodies (Cell Signaling Technology, Inc.). Proteins were visualized by using Image Studio Lite Ver 4.0 in LI-COR. Protein-content determination was measured by the method described by Bradford.

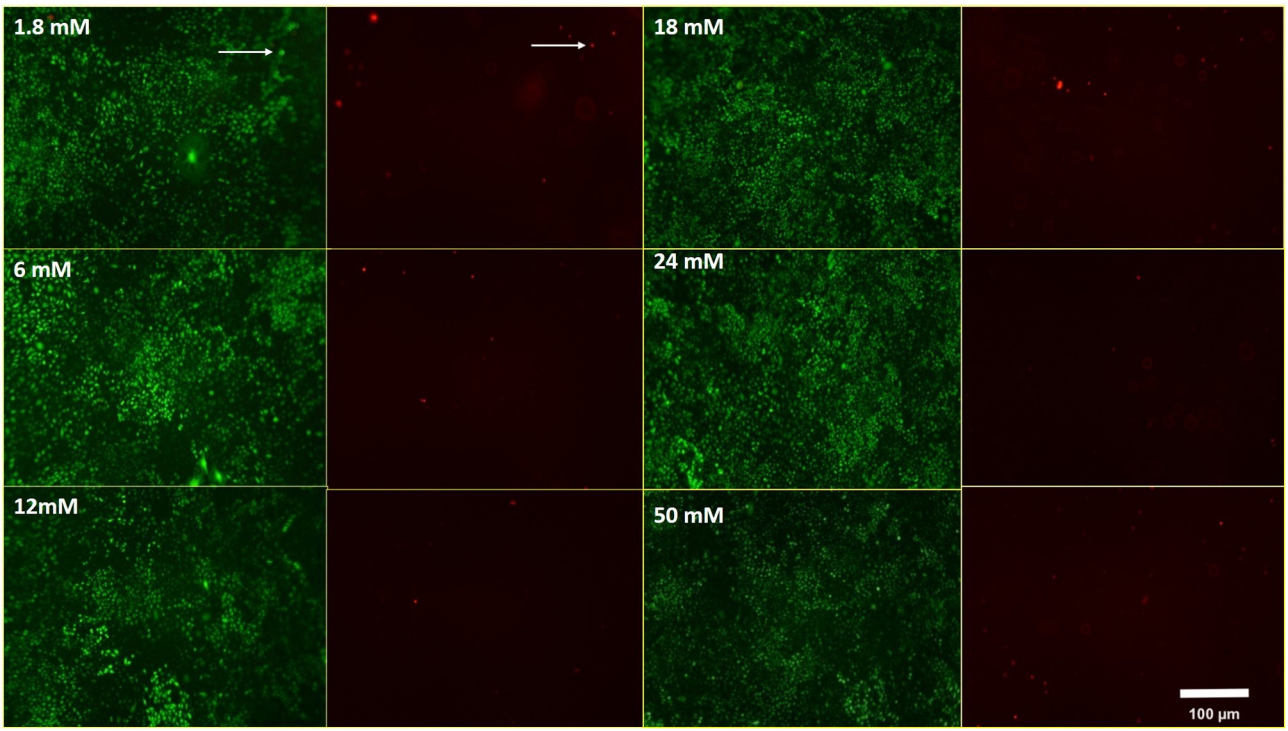

Figure 1. Live/dead cells assay at 15 min. Live/dead assay of MLO-Y4 cells incubated with different concentrations of Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 15 min. All live cells (green) on the left side and all dead cells (red) on the right side were shown with arrows.

Caspase-3 analysis. The activity of the caspase-3 was measured using a colorimetric assay (Elabscience). Centrifugation at $1x10^3$ g for 10 min was used to collect cells that had been exposed to six different concentrations of Ca^{2+} for 15 and 60 min. Lysis buffer was used to lyse the pelleted cells. Then, the cell lysates were incubated on ice for 10 min and were centrifuged at $14x10^3$ g for 1 min. Following the centrifugation, supernatants were transferred to new tubes. For measuring caspase-3 enzyme activity, 100 μl of the samples were added to the wells, after the incubation period 100 μl Biotinylated Detection Antibody working solution, 100 μl HRP conjugate working solution, 90 μl Substrate Reagent and 50 μl Stop Solution applied to each well respectively and incubated for 2 h at 37˚C in CO_2 incubator. Absorbances of the samples were read under 450 nm via the ELISA reader (Biotek).

Statistical analysis. Data are expressed as mean ± standard deviation of three independent determinations. The non-parametric approach was preferred and the Kruskal Wallis analysis of variance was used for comparison between groups. When a significant difference was detected as a result of the Kruskal Wallis test, the post hoc Dunn's test was used. In the Kruskal Wallis analysis of variance results, $P<0.05$ was accepted as significant. All analyses were performed with SPSS 25.0 (IBM SPSS Statistics 25 software (IBM Corp.) package program.

Results

The effects of Ca^{2+} concentration cell viability in MLO-Y4 cells. Since Ca^{2+} has a critical role in bone mineralization, we have designed to investigate the effect of Ca^{2+} concentration on cell viability in an experiment that consists of six different Ca^{2+} concentrations and three-time points. After MLO-Y4 cells were treated with 1.8, 6, 12, 18, 24 and 50 mM Ca^{2+} for 15 min, cell viability was determined by live/death assay. After Ca^{2+} treatment at the 15th min, there were much more live MLO-Y4 cells than dead MLO-Y4 cells in all Ca^{2+} concentrations (Fig. 1). Based on the above results, we questioned whether there was a correlation between the incubation period of Ca^{2+} and cell viability. Therefore; we set up two experiments as 60 min and 24 h at the same Ca^{2+} concentrations. Dead cells were increased at 24 and 50 mM Ca^{2+} concentrations in 60 min (Fig. 2). In 24 h of Ca^{2+} treatment, there were much more dead cells than live MLO-Y4 cells on 50 mM Ca^{2+} treatment (Fig. 3). The percentage of live/dead cells were shown in Fig. 4.

The Ca^{2+} increases CAT, SOD, GSH and NOX levels in MLO-Y4 cell lines. Ca^{2+} has important role between the free radical and enzymatic antioxidant capacity in bone metabolism. Therefore, we aimed to show how Ca^{2+} stimulation regulates the CAT, SOD, GSH and NOX levels. After MLO-Y4 cells were treated with Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 15 and 60 min, CAT, SOD, GSH and NOX levels were determined by ELISA assay.

SOD and CAT increased and reached the maximum levels in 12 mM Ca^{2+} in 15 min (Fig. 5A and B). GSH levels in different Ca^{2+} inductions did not differ significantly (Fig. 5C). In 15 min, the higher Ca^{2+} levels steadily raised NOX levels (Fig. 5D). 18, 24 and 50 mM Ca^{2+} concentrations led to the depletion of antioxidant enzymes such as CAT and SOD. The significantly increased NOX activity was observed in 18, 24 and 50 mM Ca^{2+} concentrations ($P<0.05$) (Fig. 5D). 24 mM

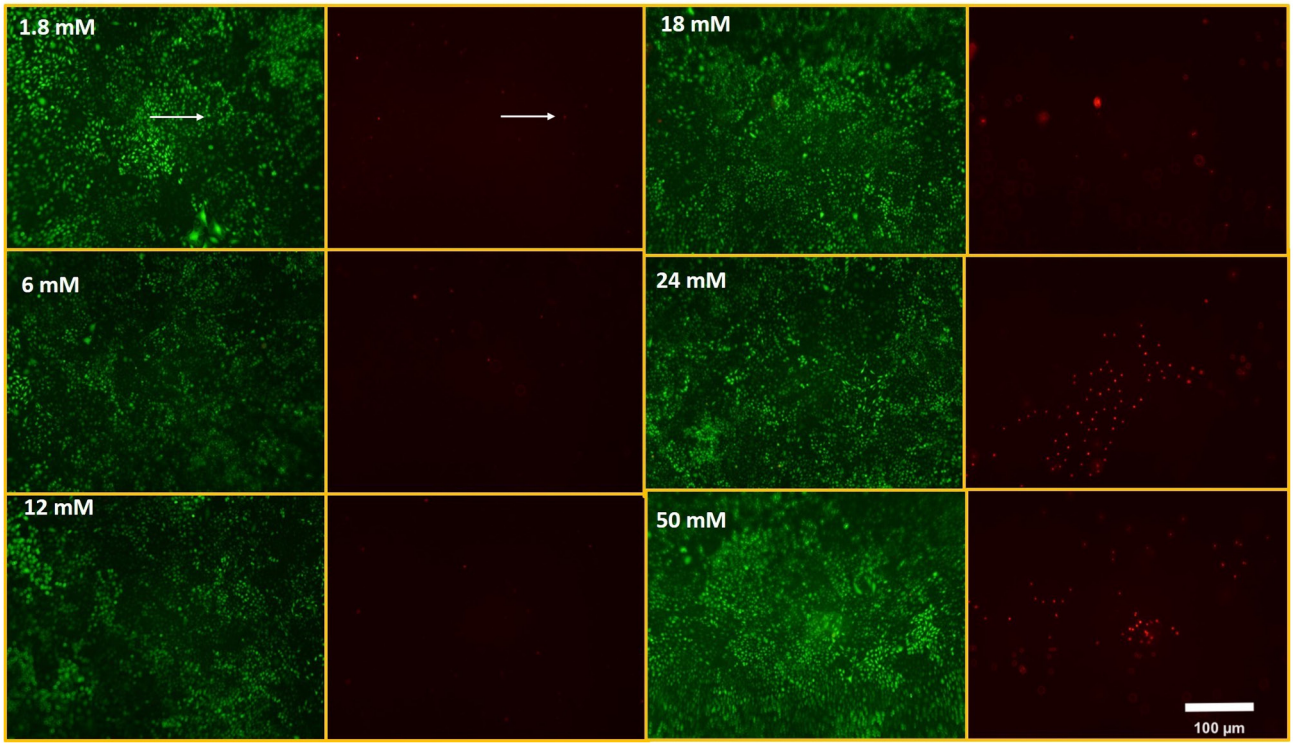

Figure 2. Live/dead cells assay at 60 min. Live/dead assay of MLO-Y4 cells incubated with different concentrations of Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 60 min. All live cells (green) on the left side and all dead cells (red) on the right side were shown with arrows.

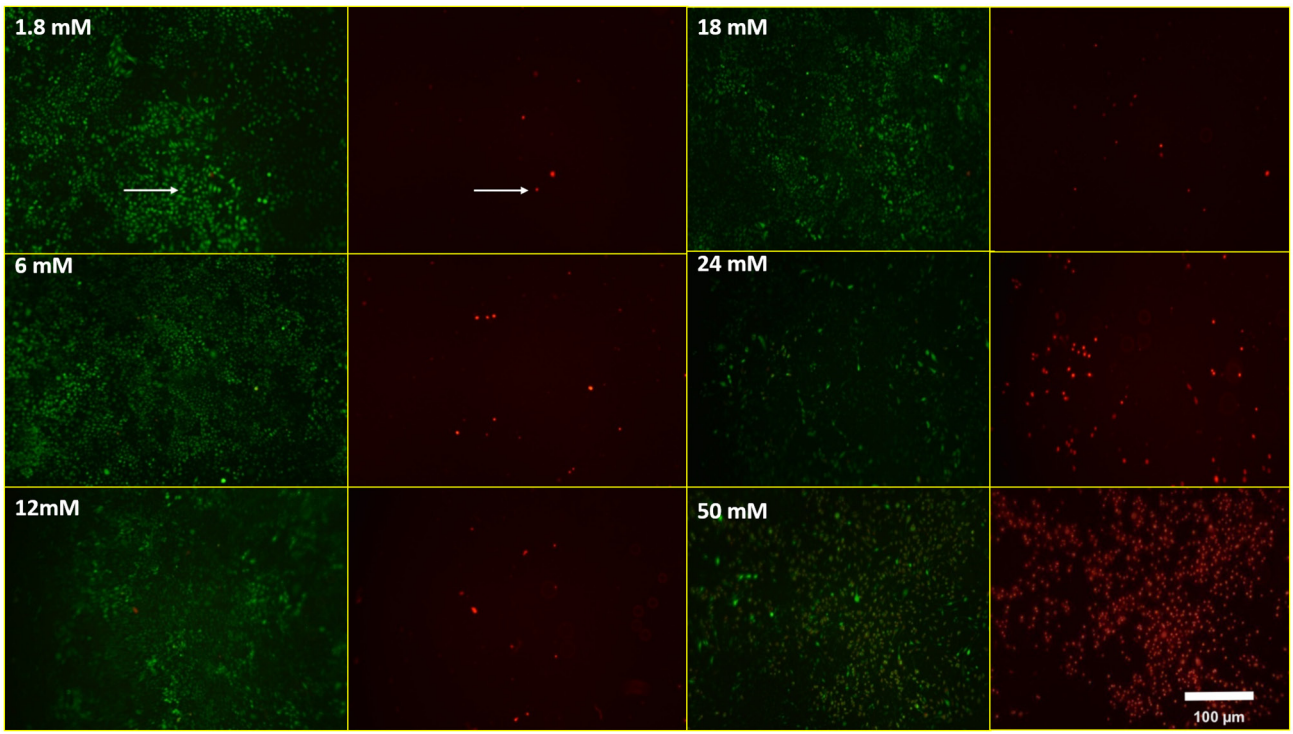

Figure 3. Live/dead cells assay at 24 h. Live/dead assay of MLO-Y4 cells incubated with different concentrations of Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 24 h. All live cells (green) on the left side and all dead cells (red) on the right side were shown with arrows.

Ca^{2+} concentration led to a significant increase in CAT levels within 60 min of treatment (Fig. 6A). SOD activity gradually increased by the elevated concentrations of Ca^{2+} in 60 min. The statistically significant difference in SOD was observed in 24 and 50 mM Ca^{2+} concentrations compared to that of 1.8 mM Ca^{2+} treatment in 60 min (Fig. 6B). There was no significant change in GSH activity in different Ca^{2+} points (Fig. 6C). NOX activity was at the highest level in 24 and

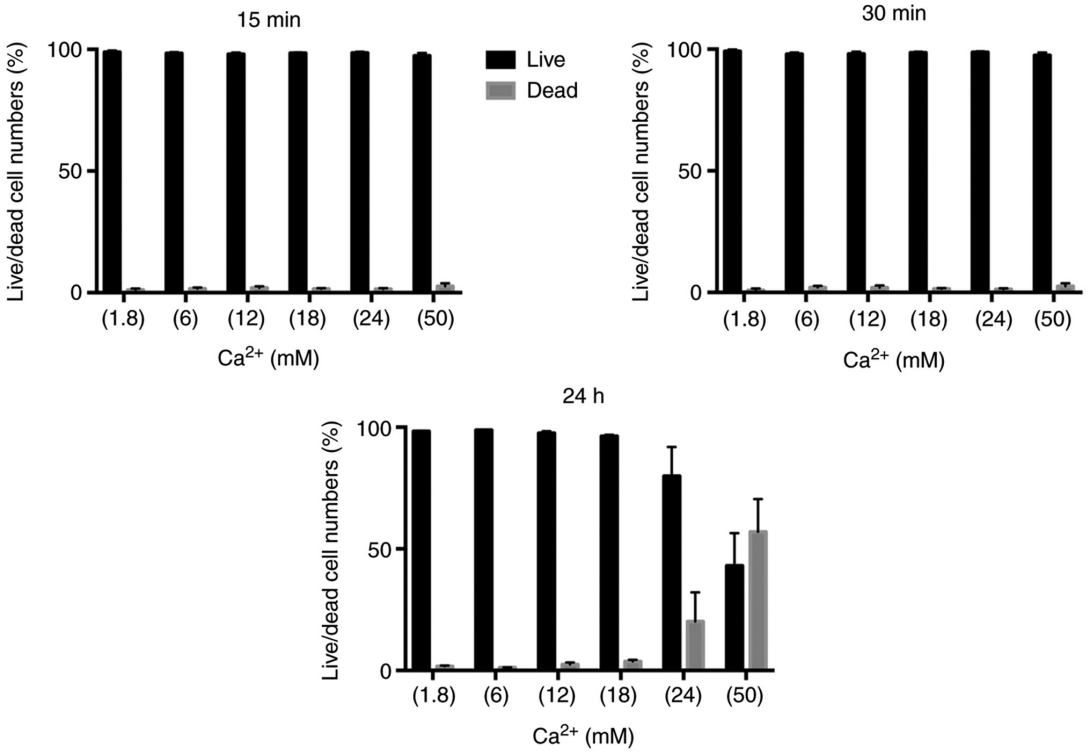

Figure 4. Percentages of live and dead cells. Percentages of live and dead cell incubated with different concentrations of Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 15 min, 60 min and 24 h.

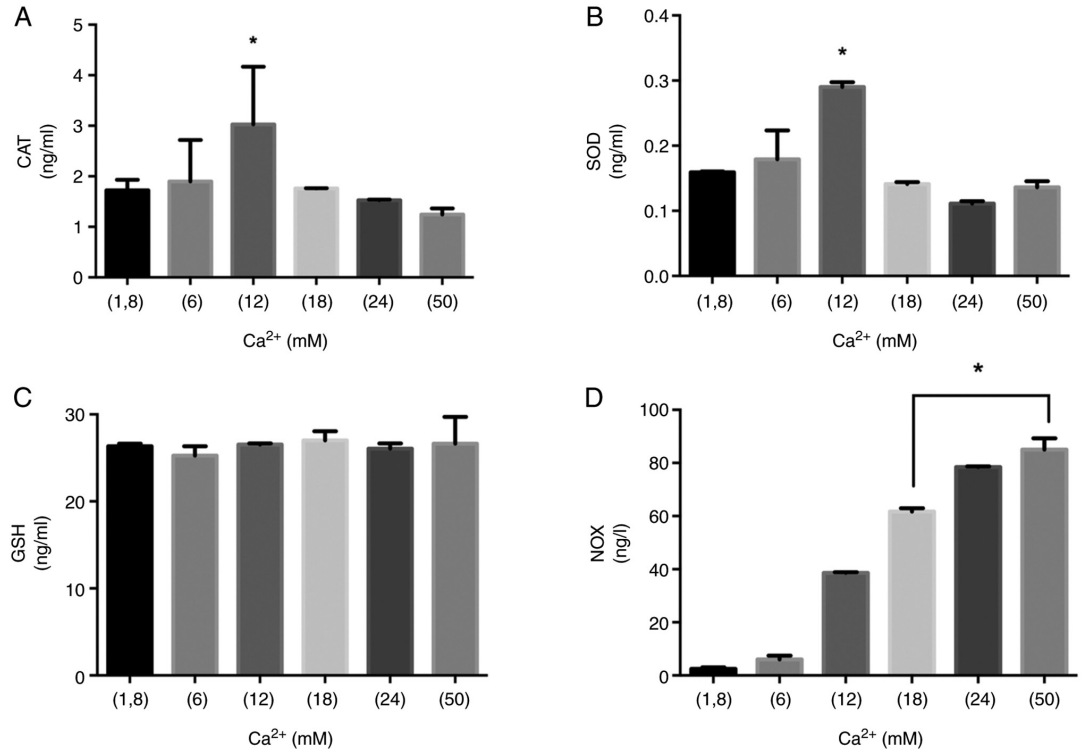

Figure 5. CAT, SOD, GSH and NOX levels at 15 min. The role of the Ca^{2+} on oxidant/antioxidant balance in MLO-Y4 at the incubation of 15 min. (A) CAT, (B) SOD, (C) GSH and (D) NOX levels were measured in MLO-Y4 cells treated with Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 15 min. *P<0.05 vs. 1.8 mM Ca^{2+} group. CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; NOX, NADPH oxidase.

50 mM compared to that of 1.8 mM Ca^{2+} concentrations in 15 and 60 min (Fig. 6D). Our results clearly show that 12 mM Ca^{2+} concentration augments CAT and SOD levels, but from the beginning of 12 mM Ca^{2+} application NOX enzyme levels significantly increased in dose dependent on manner in 15 and 60 min. 24 mM Ca^{2+} concentration significantly increased

Table I. TAS, TOS and OSI were measured in MLO-Y4 cells after 24 h of Ca^{2+} application.

Experimental group	TAS (mmol/l)	TOS (μmol/l)	OSI (arbitrary unit)
1.8 mM Ca	0.366	4.580	1.250
6 mM Ca	0.180	4.580	2.544[a]
12 mM Ca	0.252	17.328[a]	6.877[a]
18 mM Ca	0.383	18.244[a]	4.758[a]
24 mM Ca	0.149[a]	21.374[a]	14.376[a]
50 mM Ca	0.390	27.786[a]	7.130[a]

TAS and TOS were measured in MLO-Y4 cells after 24-h calcium application. OSI was measured as: OSI=[(TOS, μmol H_2O_2 equivalent/l)/(TAS, mmol Trolox equivalent/l) x10]. [a]P<0.05 compared to control (1.8 mM calcium) group. TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index.

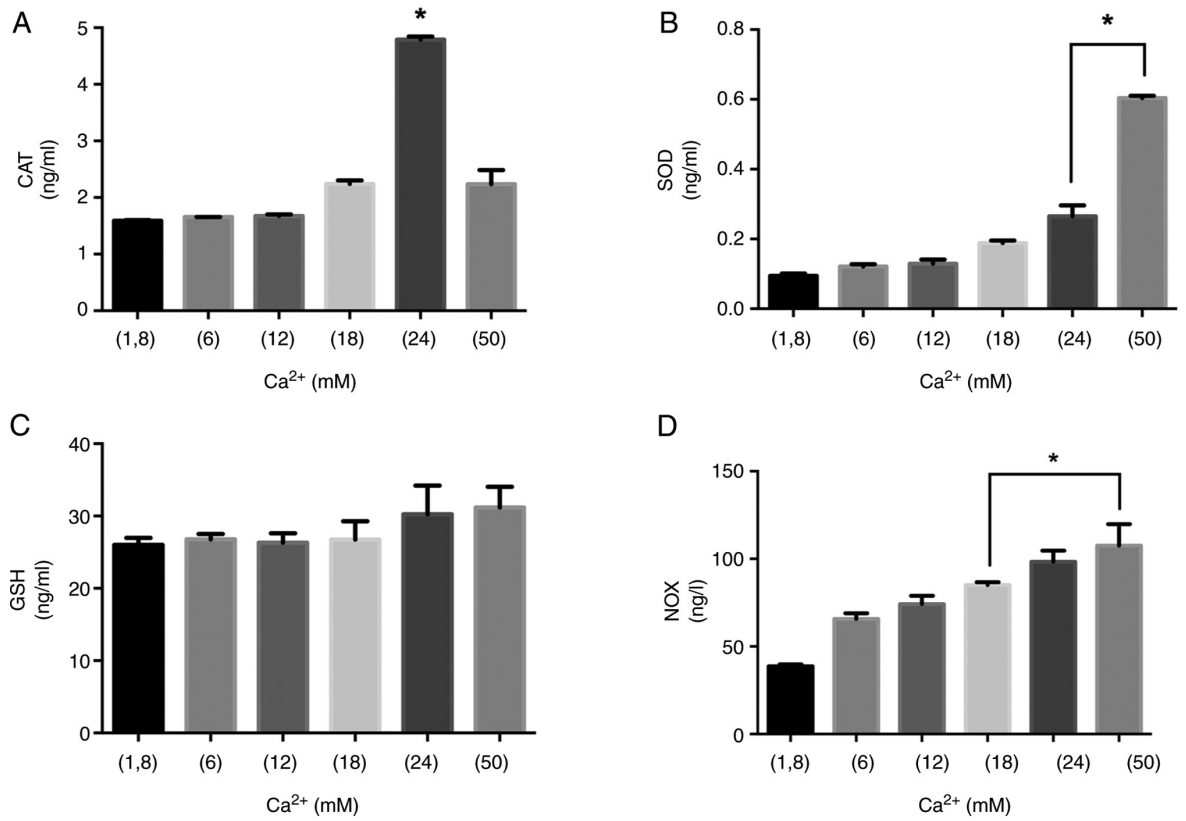

Figure 6. CAT, SOD, GSH and NOX levels at 60 min. The role of the Ca^{2+} on oxidant/antioxidant balance in MLO-Y4 at the incubation of 60 min. (A) CAT, (B) SOD, (C) GSH and (D) NOX levels were measured in MLO-Y4 cells treated with Ca^{2+} (1.8, 6, 12, 18, 24 and 50 mM) for 60 min. *P<0.05 vs. 1.8 mM Ca^{2+} group. CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; NOX, NADPH oxidase.

SOD and CAT levels in 60 min. GSH levels of MLO-Y4 cells were stable by the induction of different Ca^{2+} concentrations and different time points. NOX activity gradually increased concentration and time.

Ca^{2+} induces bone mineralization by triggering crosstalk between ROS dependent activation and PHEX, MEPE, DMP1. Previous studies have shown that ROS and/or anti-oxidant systems are related to the pathogenesis of bone loss. In our study; TAS and TOS levels were measured at the end of the 24 h of different concentrations of Ca^{2+} application, and OSI was calculated (Table I). While the amount of Ca^{2+} increased, total oxidant status and OSI were significantly increased at 12, 18, 24 and 50 mM Ca^{2+} (P<0.05 compared to 1.8 mM Ca^{2+}).

Ca^{2+} upregulates crosstalk between PHEX, MEPE, DMP1 expression and bone mineralization. PHEX, MEPE and DMP1 play an important role in bone remodeling. Therefore, to assess the expression levels of PHEX, MEPE and DMP1 in MLO-Y4 cells, we performed Western Blot analysis. Immunoblot analysis revealed that Ca^{2+} upregulates expressions of PHEX,

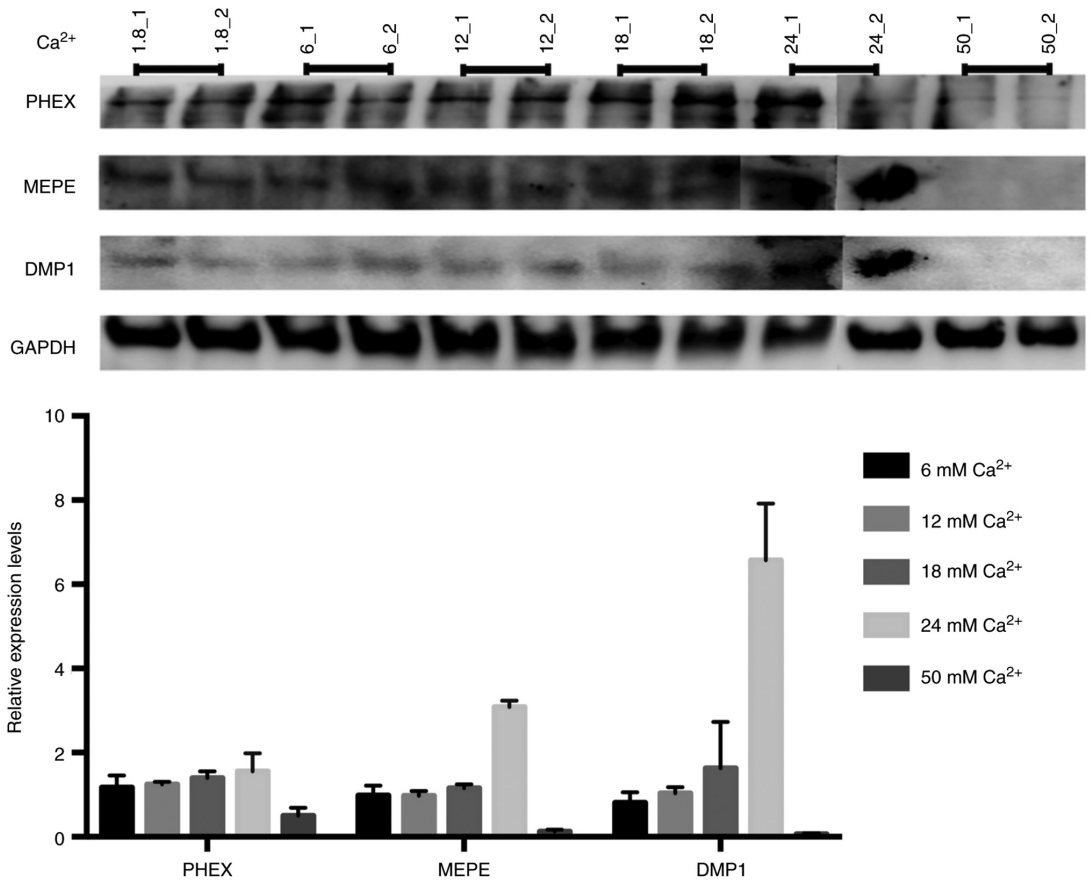

Figure 7. PHEX, DMP1 and MEPE expression levels. The Ca^{2+} promotes the PHEX, DMP1/MEPE axis. Western blotting analysis of PHEX, MEPE, DMP1 and GAPDH levels in MLO-Y4 cells treated with different concentrations of Ca^{2+}. Each Ca^{2+} concentration was analyzed densitometrically according to its GAPDH (n=2), (_1 is sample one, _2 is sample two of each Ca^{2+} concentration). Two sibling gels were prepared, run and transferred in the same electrophoresis equipment. 24_2, 50_1 and 50_2 samples were loaded to the sibling gel. PHEX, phosphate-regulating neutral endopeptidase on chromosome X; DMP1, dentin matrix protein 1; MEPE, matrix extracellular phosphoglycoprotein.

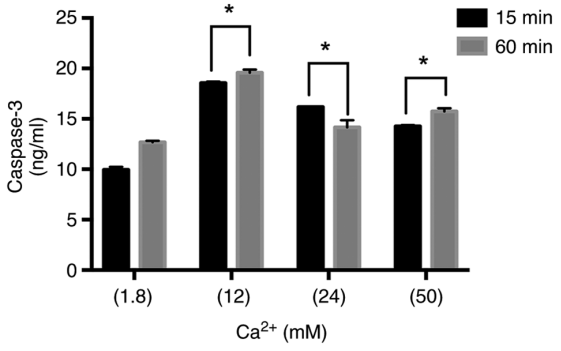

Figure 8. Caspase-3 levels at 15 and 60 min. Evaluation of caspase-3 levels of MLO-Y4. Caspase-3 levels were measured, and comparison over time (15, 60 min) was made between four groups (1.8, 12, 24 and 50 mM) significant differences between the groups vs. 1.8 mM Ca^{2+} concentration group were indicated as $^{*}P<0.05$.

MEPE and DMP1 in a dose dependent manner. The highest expression levels of PHEX, MEPE and DMP1 were seen at 24 mM Ca^{2+} treatment. We also observed a depletion/feedback in the expression of PHEX, MEPE and DMP1 at 50 mM Ca^{2+} concentration. The expression of PHEX, a pro-mineralization gene, was notably increased at the 24 mM Ca^{2+} but was suppressed at 50 mM Ca^{2+}; the highest tested concentration of Ca^{2+}. Increased exposure to Ca^{2+} appeared to alter the interaction of other osteoblast mineralization-associated genes. Our data show that optimum Ca^{2+} concentration induces bone mineralization while high Ca^{2+} concentration cause depletion of PHEX, MEPE and DMP1 expression (Fig. 7).

Caspase-3 analysis. Caspase-3 levels were studied in MLYO-4 cells grown in complete media supplemented with different concentrations of Ca^{2+} for 15 and 60 min. Caspase-3 levels were significantly elevated by the 12, 24 and 50 mM Ca^{2+} when compared to 1.8 mM Ca^{2+} treated control cells in both timelines. The rate of rising was most visible in 12 mM compared to other groups in 15 and 60 min. Incubation of MLO-Y4 cells with 1.8, 12, 24 and 50 mM of Ca^{2+} for 60 min led to increased caspase-3 levels compared to the 15 min treatment for each (Fig. 8). The data obtained from our results show all (12, 24 and 50 mM) Ca^{2+} concentrations stimulated the caspase-3 activity which is the key factor for the initiation of the apoptotic pathway.

Discussion

Calcium plays an important role in many basic biological processes like the muscle system, neural system, enzyme activity, hormone metabolism, and membrane permeability.

Moreover, Ca^{2+} is an important structural component of the skeleton. Additionally, control of Ca^{2+} ion in the extracellular fluid is vital for many metabolic activities and some endocrine control systems have developed to maintain constant Ca^{2+} concentration (23). Additionally, the breakdown of mesoporous bioactive glass scaffold releases active substances like calcium that have the potential to increase gene expression and cause osteoblast development and they also suggested that electrical stimulation could encourage cellular differentiation by controlling calcium ionic pathways and increasing calcium ion inflow into cells (24,25). Total calcium levels in healthy persons are normally kept within the range of 2.2-2.6 mM, while ionized calcium levels are usually maintained between 1.1-1.3 mM. However, soluble Ca^{2+} levels in the bone interstitial fluid may be higher, since previous studies demonstrated that the levels of Ca^{2+} ranged from 8 to 40 mM at the nearest area of the resorbing osteoclasts (26,27). Ca^{2+}concentrations can range from 8 to 40 mM in the microenvironment of the resorbing osteoclast. Osteocytes can also release considerable quantities of Ca^{2+}, as seen during lactation, implying that they are exposed to high extracellular Ca^{2+} concentrations (27). Elevated serum free calcium concentrations may have an impact on the maximal calcium concentration in the interstitial fluid of the bone that is closely next to osteoclasts. According to Sugimoto *et al*, bone remodeling depends on the presence of mononuclear cells and a high calcium concentration at the resorptive site (28).

Our results indicate that Ca^{2+} concentration significantly inhibits cell viability in a dose and time-dependent manner (Figs. 1-3). Changes in Ca^{2+} concentration have been shown to promote the function and cooperation of the osteocytes and osteoblasts (29). It has also been shown that many biological activities such as muscle contraction and nerve conduction are regulated as a result of these changes in Ca^{2+} concentration, which occurs outside the cell, especially in the presence of mechanical stress. Osteocytes are derived from the mature osteoblasts that have a more differentiated morphology during the bone formation process and are embedded in the matrix (30). The osteocytes contact with adjacent cells and communicate via dendritic processes in the canaliculi in the mineralized matrix of the bone. Furthermore, osteocytes can communicate with other cells by secreting factors into the bone marrow space or straight into the bloodstream (31,32). Cellular oxidative damage is a basic general mechanism of cell damage and is mainly caused by reactive oxygen species (ROS). While ROS is overproduced, it can cause oxidative stress which is characterized as an imbalance between ROS formation and antioxidant defense systems (33) and NADPH oxidases (NOX) produce ROS in a controlled to specifically regulate signal transduction (34). Oxidative stress can affect age-related diseases, including osteoporosis and significantly affect osteocytes. Increasing concentrations of cytoplasmic Ca^{2+} causes Ca^{2+} influx into mitochondria and Ca^{2+} disrupts metabolism of the cell. Additionally, Ca^{2+} in the cytoplasm can regulate phosphorylation/dephosphorylation of proteins (35). The lack of an impact of Ca channel inhibitors on expression levels of PHEX, MEPE and DMP1 proteins, SOD, CAT, GSH, NOX and Caspase-3 levels and total antioxidant and oxidant status is a limitation of our work. A further *in vivo* examination is necessary to determine the effect of high serum calcium concentrations on bone remodeling, which is another limitation of our work.

Cellular oxidative stress disrupts bone remodeling and leads to an imbalance between osteoclast and osteoblast activity. Previous studies have shown that ROS and/or antioxidant systems are related to the pathogenesis of bone loss (36). Osteoporosis is a metabolic bone disease characterized by low bone mineral density and a loss in bone mass, making the bone fragile and prone to fracture (37). According to a recent review article by Domazetovic *et al*, few data have shown how the molecular mechanism changes with apoptosis and oxidative status (38) but, recently, more studies have been aiming to explain the details.

In the present study, antioxidant-oxidant (CAT, SOD, GSH and NOX) enzyme levels were measured after 15 and 60 min of Ca^{2+} administration in MLO-Y4 cells (Figs. 5 and 6). Additionally, ROS induces the apoptosis of osteoblasts and supports osteoclastogenesis (39). Advanced oxidation products had been shown to inhibit the proliferation and differentiation of rat osteoblastic cells and rat mesenchymal stem cells (40) but osteocytes have been reported as a major source of the cytokine receptor activator of nuclear factor kappa-B ligand (RANKL), its functions as a key factor for osteoclast differentiation and activation (41). Yu *et al* reported that the treatment with advanced oxidation protein products significantly triggered apoptosis of MLO-Y4 cells and induced the phosphorylation of c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases. Their findings cumulatively suggest that the advanced oxidation protein products induced activation of JNK/p38 MAPK is reactive oxygen species (ROS)-dependent (42). Superoxide is formed when oxygen acquires an additional electron and oxygen is generally converted by the superoxide dismutase (SOD) which is the first enzymatic structure of the antioxidant defense system (43). Kobayashi *et al* revealed that mitochondrial superoxide dismutase 2 (*Sod2*) depletion in osteocytes improved the production of cellular superoxide and Sod2 loss decreased the number of live osteocytes inside the lucano-canalicular networks (44). To estimate oxidative and antioxidative status in cells, TOS and TAS are generally used. While Ca^{2+} concentration was increased, TOS and the OSI values also increased in our findings (Table I). Glutathione (GSH) is a tripeptide in living organisms and it acts as an antioxidant either directly by interacting with ROS (45). There was no significant difference between GSH levels of different Ca^{2+} concentrations. CAT is the one of the most important antioxidant enzymes which break down two H_2O_2 molecules into one molecule of oxygen and two molecules of water (46). Increased concentrations of Ca^{2+} in 15 min treatments led to elevated CAT activity. Previous studies suggested that concentrations of Ca^{2+} in the range of 10-15 mM demonstrated a moderate negative effect on cell survival in calvarial cells of the newborn mouse (26). Consistent with these findings, our study demonstrates that 12, 24 and 50 mM Ca^{2+} concentrations result in elevated caspase-3 levels osteocytes are sensitive to the extracellular concentration of Ca^{2+}, up to around 12 mM in 15 and 60 min of induction (Fig. 7). PHEX, MEPE and DMP1 are essential proteins released from osteocytes and play a significant role in bone mineralization. Topically administered Ca^{2+} to osteocytes in varying amounts could have a variety of effects. The goal of our study was to reveal the changes

in bone mineralization-related protein levels in different Ca^{2+} concentrations and at different time points. Our results showed that PHEX, MEPE, and DMP1 expression levels correlated to the increased concentrations of Ca^{2+} (6, 12, 18 mM). The highest increase in PHEX, MEPE and DMP1 expressions was observed at a Ca^{2+} concentration of 24 mM (Fig. 7). However, the expression levels of the proteins decreased at 50 mM Ca^{2+} concentration because of the enhanced dead cell ratio.

Some important clinical problems arise in deficiency or inactivation of PHEX and MEPE and it is important to understand the metabolism of these proteins. The inactivation of PHEX or/and DMP1 has been shown to result in an increased fibroblast growth factor 23 (FGF23) (47), and they play a key role in the progression of hypophosphatemic conditions such as tumor-induced osteomalacia and X-linked hypophosphatemic rickets/osteomalacia. PHEX deficiency results in the overproduction of fibroblast growth factor 23 (FGF23) in osteocytes (48), which leads to hypophosphatemia and impaired vitamin D metabolism (47). Miyagawa *et al* noted that DMP1 expression in osteocytic cells was increased after the 24 h treatment with 10 mM phosphate (47). Both PHEX and DMP1 are also important proteins on phosphate homeostasis. Because another role of osteocytes is to regulate phosphate (Pi) homeostasis through the release of several molecules such as PHEX, MEPE, DMP1 and FGF23 (49). DMP1 and PHEX inhibit the expression of FGF23, whereas MEPE increases it, although the mechanisms are still unknown (50,51). Both DMP1 and PHEX are excessively expressed in osteocytes and might regulate the maturation of the osteocyte cells via FGF23 regulation of phosphate homeostasis (8). Magne *et al* cultured M2H4 cells and supplemented the media with 3 mM Pi from day 8 to 20 (20). They have shown that M2H4 cells had more mineralized the dentin extracellular matrix than control conditions (1 mM Pi) and medium supplemented 3 mM Pi accumulate Ca^{2+} in cell layers (20). In our study, both expressions of PHEX and MEPE increased at 24 mM Ca^{2+} induced.

Increased levels of both PHEX and MEPE in the treatment of 24 mM Ca^{2+} are important for osteocytes metabolism and they might be used in bone treatment in the future. PHEX, MEPE, and DMP1 have also been demonstrated to be controlled during loading and unloading settings in previous investigations (52-54). These proteins involved in bone construction are expected to be upregulated in response to anabolic loading, and they are responsible for absorption (55). Gluhak-Heinrich *et al* (52) revealed that alveolar osteocytes showed high basal levels of MEPE that decreased during the first day of loading compared to that of DMP1. Kulkarni *et al* (56) have shown that mechanical loading upregulated gene expression of MEPE but not PHEX in MLO-Y4 cells during 1 and 6 h pulsating fluid flow. RANKL plays an important role during the loading/unloading mechanism because unloading increases RANKL which is an essential promoter for osteocytes (57). Osteocytes has dendritic process, which is associated with mechanical stimulation, and this process is regulated by E11 which is the earliest osteocytes protein increased by mechanical loading by osteocytes (58). Moreover, E11, MEPE and DMP1 have higher levels of expression in basal bone than in alveolar bone (59). During the skeleton is exposed to loading, an increase in these proteins secreted from osteocytes can be expected, which is very important in the mineralization of bone tissue. A new strategy for bone treatment can be designed by giving the appropriate Ca^{2+} concentration to increase the release of these proteins from osteocytes.

In a summary, our results suggest that high Ca^{2+} concentrations can perturb osteocyte cell hemostasis which leads to tissue damage and apoptosis of the bone and result in metabolic bone diseases such as osteoporosis. 24 mM Ca^{2+} concentration can trigger bone mineralization markers such as PHEX, MEPE and DMP1 can be a convenient dosage for the treatment of bone damage.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BOD and OA designed all experimental procedures of the study, and BOD conducted cell culture and the live/dead cells assay. ACD and ERK conducted CAT, SOD, GSH and NOX measurements, determination of TAS and TOS, immunoblotting for PHEX, MEPE, DMP1 and GAPDH, and caspase-3 analysis. ACD and ERK confirm the authenticity of all the raw data. JC calculated the percentage of live and dead cells. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

References

1. Palumbo C and Ferretti M: The osteocyte: From 'Prisoner' to 'Orchestrator'. J Funct Morphol Kinesiol 6: 28, 2021.
2. Florencio-Silva R, da Silva Sasso GR, Sasso-Cerri E, Simões J and Cerri PS: Biology of bone tissue: Structure, function, and factors that influence bone cells. Biomed Res Int 2015: 421746, 2015.
3. Kato Y, Windle JJ, Koop BA, Mundy GR and Bonewald LF: Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res 12: 2014-2023, 1997.
4. Robling AG and LF: Bonewald the osteocyte: New insights. Annu Rev Physiol 82: 485-506, 2020.

5. Kao RS, Abbott MJ, Louie A, O'Carroll D, Lu W and Nissenson R: Constitutive protein kinase A activity in osteocytes and late osteoblasts produces an anabolic effect on bone. Bone 55: 277-287, 2013.
6. Fisher LW and Fedarko NS: Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. Connect Tissue Res 44 (Suppl 1): S33-S40, 2003.
7. Guo D, Keightley A, Guthrie J, Veno PA, Harris SE and Bonewald LF: Identification of osteocyte-selective proteins. Proteomics 10: 3688-3698, 2010.
8. Lu Y, Yuan B, Qin C, Cao Z, Xie Y, Dallas SL, McKee MD, Drezner MK, Bonewald LF and Feng JQ: The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment. J Bone Miner Res 26: 331-340, 2011.
9. Siyam A, Wang S, Qin C, Mues G, Stevens R, D'Souza RN and Lu Y: Nuclear localization of DMP1 proteins suggests a role in intracellular signaling. Biochem Biophys Res Commun 424: 641-646, 2012.
10. Ling Y, Rios HF, Myers ER, Lu Y, Feng JQ and Boskey AL: DMP1 depletion decreases bone mineralization in vivo: An FTIR imaging analysis. J Bone Miner Res 20: 2169-2177, 2005.
11. Rowe PS, de Zoysa PA, Dong R, Wang HR, White KE, Econs MJ and Oudet CL: MEPE, a new gene expressed in bone marrow and tumors causing osteomalacia. Genomics 67: 54-68, 2000.
12. David V, Martin A, Hedge AM and Rowe PSN: Matrix extracellular phosphoglycoprotein (MEPE) is a new bone renal hormone and vascularization modulator. Endocrinology 150: 4012-4023, 2009.
13. Lu C, Huang S, Miclau T, Helms JA and Colnot C: Mepe is expressed during skeletal development and regeneration. Histochem Cell Biol 121: 493-499, 2004.
14. Jung H, Mbimba T, Unal M and Akkus O: Repetitive short-span application of extracellular calcium is osteopromotive to osteoprogenitor cells. J Tissue Eng Regen Med 12: e1349-e1359, 2018.
15. Jung H, Best M and Akkus O: Microdamage induced calcium efflux from bone matrix activates intracellular calcium signaling in osteoblasts via L-type and T-type voltage-gated calcium channels. Bone 76: 88-96, 2015.
16. Yamauchi M, Yamaguchi T, Kaji H, Sugimoto T and Chihara K: Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells. Am J Physiol Endocrinol Metab 288: E608-E616, 2005.
17. Boskey AL and Roy R: Cell culture systems for studies of bone and tooth mineralization. Chem Rev 108: 4716-4733, 2008.
18. Bonewald LF, Harris SE, Rosser J, Dallas MR, Dallas SL, Camacho NP, Boyan B and Boskey A: Von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation. Calcif Tissue Int 72: 537-547, 2003.
19. Zhou H, Wu T, Dong X, Wang Q and Shen J: Adsorption mechanism of BMP-7 on hydroxyapatite (001) surfaces. Biochem Biophys Res Commun 361: 91-96, 2007.
20. Magne D, Bluteau G, Lopez-Cazaux S, Weiss P, Pilet P, Ritchie HH, Daculsi G and Guicheux J: Development of an odontoblast in vitro model to study dentin mineralization. Connect Tissue Res 45: 101-108, 2004.
21. Price PA, June HH, Hamlin NJ and Williamson MK: Evidence for a serum factor that initiates the re-calcification of demineralized bone. J Biol Chem 279: 19169-19180, 2004.
22. Erel O: A new automated colorimetric method for measuring total oxidant status. Clin Biochem 38: 1103-1111, 2005.
23. Sheweita SA and Khoshhal KI: Calcium metabolism and oxidative stress in bone fractures: Role of antioxidants. Curr Drug Metab 8: 519-525, 2007.
24. Shuai C, Liu G, Yang Y, Qi F, Peng S, Yang W, He C, Wang G and Qian G: A strawberry-like Ag-decorated barium titanate enhances piezoelectric and antibacterial activities of polymer scaffold. Nano Energy 74: 104825, 2000.
25. Shuaia C, Xu Y, Feng P, Wang G, Xiong S and Peng S: Antibacterial polymer scaffold based on mesoporous bioactive glass loaded with in situ grown silver. Chemical Engineering J 374: 304-345, 2019.
26. Maeno S, Niki Y, Matsumoto H, Morioka H, Yatabe T, Funayama A, Toyama Y, Taguchi T and Tanaka J: The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. Biomaterials 26: 4847-4855, 2005.
27. Welldon KJ, Findlay DM, Evdokiou A, Ormsby RT and Atkins GJ: Calcium induces pro-anabolic effects on human primary osteoblasts associated with acquisition of mature osteocyte markers. Mol Cell Endocrinol 376: 85-92, 2013.
28. Sugimoto T, Kanatani M, Kano J, Kaji H, Tsukamoto T, Yamaguchi T, Fukase M and Chihara K: Effects of high calcium concentration on the functions and interactions of osteoblastic cells and monocytes and on the formation of osteoclast-like cells. J Bone Miner Res 8: 1445-1452, 1993.
29. Dvorak MM, Siddiqua A, Ward DT, Carter DH, Dallas SL, Nemeth EF and Riccardi D: Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. Proc Natl Acad Sci USA 101: 5140-5145, 2004.
30. Mullen CA, Haugh MG, Schaffler MB, Majeska RG and McNamara LM: Osteocyte differentiation is regulated by extracellular matrix stiffness and intercellular separation. J Mech Behav Biomed Mater 28: 183-194, 2013.
31. Fulzele K, Lai F, Dedic C, Saini V, Uda Y, Shi C, Tuck P, Aronson JL, Liu X, Spatz JM, *et al*: Osteocyte-Secreted Wnt Signaling Inhibitor Sclerostin Contributes to Beige Adipogenesis in Peripheral Fat Depots. J Bone Miner Res 32: 373-384, 2017.
32. Uda Y, Azab E, Sun N, Shi C and Pajevic PD: Osteocyte mechanobiology. Curr Osteoporos Rep 15: 318-325, 2017.
33. Sarban S, Kocyigit A, Yazar M and Isikan UE: Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. Clin Biochem 38: 981-986, 2005.
34. Schroder K: NADPH oxidases in bone homeostasis and osteoporosis. Free Radic Biol Med 132: 67-72, 2019.
35. Ermak G and Davies KJ: Calcium and oxidative stress: From cell signaling to cell death. Mol Immunol 38: 713-721, 2002.
36. Ostman B, Michaëlsson K, Helmersson J, Byberg L, Gedeborg R, Melhus H and Basu S: Oxidative stress and bone mineral density in elderly men: Antioxidant activity of alpha-tocopherol. Free Radic Biol Med 47: 668-673, 2009.
37. Banfi G, Iorio EL and Corsi MM: Oxidative stress, free radicals and bone remodeling. Clin Chem Lab Med 46: 1550-1555, 2008.
38. Domazetovic V, Marcucci G, Iantomasi T, Brandi ML and Vincenzini MT: Oxidative stress in bone remodeling: Role of antioxidants. Clin Cases Miner Bone Metab 14: 209-216, 2017.
39. Jilka RL, Noble B and Weinstein RS: Osteocyte apoptosis. Bone 54: 264-271, 2013.
40. Zhong ZM, Bai L and Chen JT: Advanced oxidation protein products inhibit proliferation and differentiation of rat osteoblast-like cells via NF-kappaB pathway. Cell Physiol Biochem 24: 105-114, 2009.
41. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, *et al*: Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17: 1231-1234, 2011.
42. Yu C, Huang D, Wang K, Lin B, Liu Y, Liu S, Wu W and Zhang H: Advanced oxidation protein products induce apoptosis, and upregulate sclerostin and RANKL expression, in osteocytic MLO-Y4 cells via JNK/p38 MAPK activation. Mol Med Rep 15: 543-550, 2017.
43. Wang Y, Branicky R, Noë A and Hekimi S: Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J Cell Biol 217: 1915-1928, 2018.
44. Kobayashi K, Nojiri H, Saita Y, Morikawa D, Ozawa Y, Watanabe K, Koike M, Asou Y, Shirasawa T, Yokote K, *et al*: Mitochondrial superoxide in osteocytes perturbs canalicular networks in the setting of age-related osteoporosis. Sci Rep 5: 9148, 2015.
45. Lushchak VI: Glutathione homeostasis and functions: Potential targets for medical interventions. J Amino Acids 2012: 736837, 2012.
46. Nandi A, Yan LY, Jana CK and Das N: Role of catalase in oxidative stress- and age-associated degenerative diseases. Oxid Med Cell Longev 2019: 9613090, 2019.
47. Miyagawa K, Yamazaki M, Kawai M, Nishino J, Koshimizu T, Ohata Y, Tachikawa K, Mikuni-Takagaki Y, Kogo M, Ozono K and Michigami T: Dysregulated gene expression in the primary osteoblasts and osteocytes isolated from hypophosphatemic Hyp mice. PLoS One 9: e93840, 2014.
48. Wang X, Wang S, Li C, Gao T, Liu Y, Rangiani A, Sun Y, Hao J, George A, Lu Y, *et al*: Inactivation of a novel FGF23 regulator, FAM20C, leads to hypophosphatemic rickets in mice. PLoS Genet 8: e1002708, 2012.
49. Bellido T: Osteocyte-driven bone remodeling. Calcif Tissue Int 94: 25-34, 2014.
50. Martin A, Liu S, David V, Li H, Karydis A, Feng JQ and Quarles LD: Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling. FASEB J 25: 2551-2562, 2011.

51. Lavi-Moshayoff V, Wasserman G, Meir T, Silver J and Naveh-Many T: PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: A bone parathyroid feedback loop. Am J Physiol Renal Physiol 299: F882-F889, 2010.
52. Gluhak-Heinrich J, Pavlin D, Yang W, MacDougall M and Harris SE: MEPE expression in osteocytes during orthodontic tooth movement. Arch Oral Biol 52: 684-690, 2007.
53. Yang W, Lu Y, Kalajzic I, Guo D, Harris MA, Gluhak-Heinrich J, Kotha S, Bonewald LF, Feng JQ, Rowe DW, *et al*: Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. J Biol Chem 280: 20680-20690, 2005.
54. Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE and Pavlin D: Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo. J Bone Miner Res 18: 807-817, 2003.
55. Bonewald LF: The role of the osteocyte in bone and nonbone disease. Endocrinol Metab Clin North Am 46: 1-18, 2017.
56. Kulkarni RN, Bakker AD, Everts V and Klein-Nulend J: Inhibition of osteoclastogenesis by mechanically loaded osteocytes: involvement of MEPE. Calcif Tissue Int 87: 461-468, 2010.
57. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC and O'Brien CA: Matrix-embedded cells control osteoclast formation. Nat Med 17: 1235-1241, 2011.
58. Zhang K, Barragan-Adjemian C, Ye L, Kotha S, Dallas M, Lu Y, Zhao S, Harris M, Harris SE, Feng JQ and Bonewald LF: E11/gp38 selective expression in osteocytes: Regulation by mechanical strain and role in dendrite elongation. Mol Cell Biol 26: 4539-4552, 2006.
59. Zakhary I, Alotibi F, Lewis J, ElSalanty M, Wenger K, Sharawy M and Messer RLW: Inherent physical characteristics and gene expression differences between alveolar and basal bones. Oral Surg Oral Med Oral Pathol Oral Radiol 122: 35-42, 2016.

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