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ORIGINAL ARTICLE

Osteoclast-like activity of U937 cells, peripheral blood mononuclear cells, and periodontal ligament fibroblasts subjected to mechanical stress by centrifugal force

Pedro Moreno[1], Angie Bedoya-Rodriguez[1], Bibiana Pazos[2], Juan Sebastián Bedoya[2], Orlando Acosta[1], Carlos Guerrero*[1]

Edited by
Juan Carlos Salcedo-Reyes
salcedo.juan@javeriana.edu.co

1. Department of Physiological Sciences, Faculty of Medicine, Universidad Nacional de Colombia, Bogota, D.C., Colombia.

2. Department of Orthodontics, Faculty of Dentistry, Universidad Nacional de Colombia.

*caguerrerof@unal.edu.co

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Abstract

Several *in vitro* models have investigated the consequences of mechanical stimuli on osteoclasts (OCs). However, the mechanisms whereby mechanical forces trigger osteoclast responses remain poorly understood, and the generation of reactive oxygen species (ROS) and their relationship with bone resorption in OCs under the influence of mechanical forces require investigation. The present work examined the role of centrifugal force application on ROS production and its effect on osteoclast activity and differentiation. Human U937 macrophage cells, peripheral blood mononuclear cells (PBMCs), and periodontal ligament (PDL) fibroblasts treated with polyethylene glycol (PEG) or N-acetylcysteine (NAC) were subject to centrifugal force. Osteoclast markers such as tartrate-resistant acid phosphatase (TRAP) and bone resorption activities were measured. ROS levels and actin ring formation were determined. Also, U937 cell responses to centrifugal force and PEG-induced fusion were studied. Individual cells subjected to centrifugal exertion increased their ROS levels, formed actin-like rings, revealed TRAP expression and bone resorption activities, and expressed typical osteoclast markers. Control PEG-fused U937 cells also showed these effects, and cell treatment with NAC stopped all these responses. Centrifugal force, as well as PEG-induced cell fusion, can promote osteoclast-like features, including oxidative stress. The present experimental model allowed us to understand the mechanisms underlying the osteoclast differentiation associated with ROS production stimulated by mechanical compressive force, where NAC can contribute to reducing this oxidative stress condition.

Keywords: bone resorption; centrifugal force; monocyte fusion; periodontal ligament; osteoclast activity; polyethylene glycol.

1. Introduction

Bone remodeling is a physiological process supporting skeletal structure and underlying orthodontic tooth movement (OTM). Orthodontic procedures involve compressive and tensional forces on the periodontium [1]. Orthodontic loading can change the stress distribution of the periodontal ligament (PDL) by altering the organization and remodeling of the collagen fibers located in the extracellular matrix (ECM) of PDL [2]. The theory of OTM poses that teeth move in two phases: A catabolic phase involving bone resorption by osteoclasts at both compression and tension sites and an anabolic phase to restore the alveolar bone to its pretreatment levels [3].

Many studies have addressed the mechanisms of mechanical signals transduction into biological signals that regulate bone homeostasis through PDL fibroblasts during orthodontic treatment [4]. PDL fibroblasts influence osteoclastogenesis from bone marrow-derived macrophages (BMM) following the application of tension or compression forces and involving TNF-α-mediated activation of $CD4^+$ T cells [5]. Applying compressive forces on PDL fibroblasts can induce metabolic phases to express and release asporin, inhibiting bone formation during orthodontic tooth movement. Continuous compression can induce the fusion and differentiation of RAW264.7 cells into $TRAP^+$ multinucleate cells by expressing dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) and stimulating the nuclear transport of nuclear factor-activated T cells c1 (NFATc1) [6].

Osteoclasts (OCs) are multinucleated cells (polykarions) formed by the fusion of mononuclear precursors derived from the monocyte-macrophage lineage [7]. The receptor activator of nuclear factor-κB ligand (RANKL) and the macrophage colony-stimulating factor (M-CSF) pathways are necessary and sufficient for OCs differentiation. RANKL, secreted by osteoblasts (OBs) and PDL fibroblasts, stimulates bone resorption by binding to its receptor (RANK) and inducing OCs formation from hematopoietic precursors [8].

Increased OCs activity contributes to pathological bone resorption, such as osteoporosis and periodontitis, and physiological conditions, such as OTM. Mechanical stress on bone plays a central role in maintaining bone homeostasis [9]. Several in vitro models describe the effect of mechanical stimuli on OCs, differing in their analytical techniques and methodologies. These models include the direct effect of mechanical stimuli on the behavior of osteoclast-like cells, substrate or scaffold deformation on which the cells are grown [10], fluid flow shear stress-induced formation of osteoclasts [11], and centrifugal compression applied to PDL fibroblasts. Despite all of these in vitro models being used to investigate the effects of mechanical stimuli on bone cells, the mechanisms whereby mechanical forces trigger a response on OCs and the nature of this response remain poorly understood.

No experimental model satisfactorily explains the biochemical and morphological changes of cells under sustained and mild compression. Differentiation factors, such as hormones, cytokines, and growth factors like dexamethasone, vitamin D3, M-CSF, and RANKL, were added to cultures before applying mechanical forces to assess differentiation [12]. Alternatively, osteoclast-like cells were induced by co-culturing bone marrow cells with gingival or PDL fibroblasts to evaluate OC activity [13]. Neither of these methods allowed for determining whether bone resorption, following force application, was elicited by differentiation factors or the force itself. Furthermore, the extent to which the individual factors affect the intracellular reactive oxygen species (ROS) concentration remains unknown.

ROS increase during OC differentiation, suggesting a direct relationship between osteoclastogenesis and ROS increase in hematopoietic OC precursors [14]; an oxidative environment is necessary for pre-osteoclast proliferation. This condition induces the activation of multiple signaling pathways to regulate intracellular oxidative stress and the onset of OC differentiation. However, the role of ROS in osteoclast-mediated bone resorption is poorly understood. The NF-$\kappa\beta$-pathway, which RANKL activates, is a central regulator of oxidative stress [15]. Exposure of bone marrow mononuclear cells (BMMCs) to antioxidants, such as N-acetylcysteine (NAC) or curcumin, decreases the expression of NF-$\kappa\beta$ protein, suggesting a possible inhibitory effect of antioxidants on the osteoclastogenesis process mediated through the RANKL pathway [16-18]. Treatment with antioxidants also significantly reduces the formation of actin rings, a process induced by RANKL [12]. In this way, ROS acts as a mediator of OC signaling pathways, such

as the RANKL pathway [19]. However, ROS levels have not been measured in cells to which compressive force is applied. Their relationship with the resorptive activity remains unclear. Few studies address the effects of mechanical forces applied on osteoclast precursor cells to induce osteoclast differentiation [20].

This study assessed mechanical compression effects by applying only centrifugal force to U937 cells (a human macrophage cell line), peripheral blood mononuclear cells (PBMCs), and PDL fibroblasts in the absence of differentiation factors. All these cells revealed osteoclast-like features when subjected to mechanical force. These features included ROS levels, TRAP activity, actin ring formation, bone resorptive activity, and the expression of some typical OC markers. These variables were compared with those exhibited by cells fused with polyethylene glycol (PEG), as reported previously [21, 22]. Our results revealed that cultured osteoclast-like cells obtained by either applying centrifugal force or PEG-induced cell fusion similarly showed osteoclast-like features. The osteoclast differentiation method used here opens an experimental path to study the implication of ROS during the expression of osteoclast activities induced by mechanical compression. Overall, our results suggest that the single application of a mechanical force such as that produced by centrifugal force induces osteoclast-like cell activities.

2. Materials and methods

2.1. Reagents and cells

RPMI 1640 medium and fetal calf serum (FCS) were purchased from Invitrogen/Gibco (Carlsbad, CA, USA). Antibodies against cathepsin K (CTSK) (SC-30056), calcitonin receptor (CT) (SC-20743), carbonic anhydrase II (CAII) (SC-25596), vacuolar H^+-ATPase (V-ATPase) subunit C1 (SC-14756), integrin $\beta 3$ (SC-6627), dendritic cell-specific transmembrane protein (DC-STAMP) (SC 9769), matrix metallopeptidase 9 (MMP-9) (SC 6841), ADAM12 (SC 16527), and p-NF-$\kappa\beta$ (SC 271908) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PEG 1450 was provided by Sigma (Sigma-Aldrich, St. Louis, MO, USA) and prepared at 50 % (w/v) in PBS using Sigma reagents. Bovine cortical bone slices (0.5 cm long, 0.5 cm wide, and 0.5 mm thick) were prepared as described elsewhere [21, 22]. N-acetyl-L-cysteine (NAC) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

U937 cells (ATCC CRL-1593.2) were kindly donated by Dr. C. A. Clavijo, Faculty of Sciences, Universidad Nacional de Colombia. U937 cells were cultured in RPMI 1640 medium supplemented with 10 % FCS (v/v) at 37 °C in a 5 % CO_2 atmosphere. PBMCs were obtained from healthy human subjects. PDL fibroblasts were from patients undergoing dental surgery at the Dental Service, School of Dentistry, Universidad Nacional de Colombia. All patients gave signed informed consent, and the Ethics Committee of the School of Medicine approved the study.

2.2. Isolation of PBMCs and PDL fibroblasts

Antecubital heparinized blood samples (5 ml) were drawn from healthy individuals aged 23 to 26 years following informed consent, as previously described [22]. Blood samples were diluted with 10 ml of sterile saline solution (SSS), layered onto Ficoll-Hypaque® gradients, and centrifuged at 700 g for 30 min. Isolated PBMCs were washed three times with SSS and stimulated with 10 µg ml^{-1} of phytohemagglutinin P (PHA-P) for 72 h at 37 °C in a humidified chamber containing 5 % CO_2. Cell purity and viability were assessed using standard flow cytometry and trypan blue exclusion test, respectively.

For the direct explant of PDL fibroblasts, three tissue specimens were obtained from healthy subjects aged between 20 and 35 years, as previously described [23]. Clinically healthy premolar or upper third molar, extracted for orthodontic or non-functionality reasons, were rinsed in SSS containing 2 % penicillin-streptomycin solution (100 U ml^{-1} and 100 µg ml^{-1}, respectively). After disinfection with a povidone-iodine solution for 5 min and washing in culture media, PDL explants were scrapped off the middle-third section of the root surface with a sterile surgical blade. PDL fibroblasts were cultured in RPMI 1640 medium with 10 % FCS and 2 % penicillin-streptomycin solution until cell numbers sufficed for the experiment (from three to five passages). All culture plates were examined by inverted microscopy every 24 h, and fibroblasts were grown until confluence.

2.3. Cell fusion

U937 cells, PBMCs, and PDL fibroblasts (6×10^6 each) cultured in RPMI 1640 medium containing 10 % FCS were collected by centrifugation at 700 *g* for 5 min. The supernatant was removed, and the cell pellet was covered with 50 µl of 50 % (w/v) PEG 1450 followed by gentle detachment from the tube bottom for 5 min. The cell suspension was diluted adding 400 µl of RPMI 1640 every 60 sec for 5 min. After dilution was completed, 800 µl and 1600 µl of RPMI 1640 were successively added for 5 min [22]. After the PEG solution was diluted, the cells were centrifuged for 10 min at 700 *g*, washed with RPMI 1640 medium, and resuspended in the same medium containing 10 % FCS. PEG-fused U937 cells (about 3×10^4 cells in 30 µl) in RPMI 1640 medium with or without NAC (30 mM) were placed onto bovine cortical bone slices in 24-well culture plates and incubated for 2 h at 37 °C. Following this incubation time, 500 µl of RPMI 1640 medium containing 10 % FCS were added to each well, and the PEG-fused U937 cells were harvested at 12 h, 24 h, 48 h, and 96 h.

2.4. Centrifugal exertion

U937 cells, PBMCs, and PDL fibroblasts (6×10^6 each) grown in RPMI medium containing 10 % FCS were harvested after 48 h, 72 h, and 96 h, respectively, and centrifuged at 700 *g* for 5 min. Supernatants were discarded, and cell pellets were resuspended in RPMI medium by gentle vortexing and homogenization via continuous passage through a sterile micropipette blue tip. U937 cells, PBMCs, and PDL fibroblasts at 3×10^4 cells in 30 µl of RPMI medium containing 10 % FCS were placed onto bovine cortical bone slices. Each bone slice was placed into a well of a 24-well culture plate and incubated for 2 h at 37 °C in a humidified atmosphere containing 5 % CO_2 to induce adhesion. After adhesion, 500 µl of RPMI medium containing 10 % FCS were added to each well, and the plate was placed in a horizontal rotary motor (Cole Parmer Roto Torque Heavy Duty Rotator Model 7637®, NJ USA) at a constant speed for four days in an incubator at 37 °C with a humidified atmosphere containing 5 % CO_2.

To calculate the force exerted on the cells, the following formula was applied [24]:

$$P = \frac{m \times r \times \mathrm{rpm}^2 \times \pi^2}{A \times 9.8 \times 900},$$

where P is the pressure in kg cm^{-2}; m is the mass of the medium; r is the radius of the rotary motor; rpm are the number revolutions per minute, and A is the contact area (cm^2) between the medium and the cells. The values of each variable were: $m = 0.015$ g, $r = 0.065$ m, $A = 4\,cm^2$ and rpm $= 3, 30,$ or 80, corresponding respectively to pressures (P): 2.4×10^{-6} g cm^{-2}, 2.4×10^{-4} g cm^{-2} or 1.74×10^{-3} g cm^{-2}. The rotation speeds and the corresponding pressures were classified as low, medium, and high [24]. These rotation speeds were defined considering

their effect on TRAP and bone resorptive activities without affecting cell viability. Medium centrifugal force was used in the case of cells treated or left untreated with 30 mM NAC. Cells were grown under rotation for 24 h, 72 h, and 96 h and then fixed for 40 min at 4 °C with 4 % paraformaldehyde, followed by three PBS washes (200 µl each). Then, the plates containing cells were placed for 10 min at 4 °C before adding 200 µl of ice-cold acetone/ethanol (1 : 1 v/v) and then incubated at 4 °C for 1 h. Subsequently, the TRAP and resorptive activities of cells on each bovine cortical bone slice were evaluated. Subsequently, ROS, actin rings, or cytoplasmic membrane markers on each bone slice were assessed.

2.5. ROS level evaluation

ROS formation was measured using the Cellomics Oxidative Stress 1 HCS Reagent Kit. U937 cells (3×10^4 in 30 µl RPMI) subjected to centrifugal force or PEG-induced fusion were applied onto sterile bovine cortical bone slices, incubated at 37 °C, and harvested after 12 h, 24 h, 48 h, or 96 h of incubation. The harvested cells were placed in Dulbecco's Modified Eagle Medium (DMEM), added with dihydroethidium (DHE) (1.6 µg ml^{-1} final concentration), and incubated for 30 min at 37 °C. Cells were treated with 4 % paraformaldehyde in PBS for 30 min at 37 °C, followed by three times washing with PBS before staining with Hoechst (1 µg ml^{-1}) for 5 min. Ten photographs of representative images were taken using an inverted fluorescence microscope (SOPTOP®, Kuala Lumpur Malaysia), followed by image processing and analysis using the ImageJ 1.3 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) (http://rsb.info.nih.gov/ij/). All tests were performed in duplicate and repeated twice.

2.6. TRAP staining

U937 cells (3×10^4 in 30 µl RPMI) were subjected to centrifugal force to determine the expression of TRAP activity as a biomarker of OCs. U937 cell TRAP expression was compared with that of PEG-fused U937 cells that had not been subjected to centrifugation. In a separate experiment, U937 cells (3×10^4 in 30 µl RPMI) were subjected to centrifugal force and then applied to sterile bovine cortical bone slices. A sample of PEG-fused U937 cells was also separately applied onto the same bone slices, followed by incubation at 37 °C. Bone slices containing the U937 cells were harvested at 12, 24, 48, and 96 h after incubation and fixed with acetone/ethanol as described above. Fixed cells were incubated with substrate solution consisting of Veronal buffer (0.53 % barbituric acid and 1.1 % NaOH containing 3.84 % sodium acetate, 4 % pararosaniline, 4 % sodium nitrite, and 0.0126 % naphthol AS-BI phosphate; Sigma-Aldrich, St. Louis, MO, USA). The pH of the substrate solution was adjusted to 4.3 before use. Each bovine cortical bone slice was incubated with 300 µl of substrate solution for 2 h at 37 °C. Hematoxylin (30 µl) counterstaining was performed for 30 min followed by washing with a solution containing ethanol/distilled water/acetic acid (70 : 29 : 1; v/v) and then with distilled water. The air-dry bone slices at room temperature (RT) were observed for TRAP$^+$ multinucleated cells following a "W" path. Photographs of representative images were taken using an inverted microscope (SOPTOP®, Kuala Lumpur, Malaysia). Data were expressed as TRAP$^+$ cells mm^{-2}.

2.7. Resorptive activity

After determining the proportion of TRAP-positive cells, cells were removed from bone slices with 6 % NaOCl and 5.2 % NaCl under constant agitation for 15 min. Bone slices were extensively rinsed (five times) with water under continuous agitation, then stained with Coomassie blue for 5 minutes and washed twice with methanol/acetic acid/water solution (50 : 5 : 45; v/v) for 1 min. Bone slices were submerged in distilled water and agitated for 20 sec. The number and area of

resorption pits were quantified on photographs with an external Digital Camera (Canon S70), followed by image processing and analysis with the ImageJ 1.3 software. The total area, mean pit area, and percentage of total area reabsorbed were determined.

2.8. Cell fusion analysis

U937 cells (1×10^6) were separately incubated for 30 min at RT in 1 ml DMEM containing 10 µM DiO or 10 µM DiD and then washed three times with DMEM. As described above, cells treated with DiO or DiD were mixed and then PEG-fused or subjected to centrifugal force. U937 cells (3×10^4 in 30 µl RPMI containing 10 % FCS) were subjected to centrifugal force or PEG-induced fusion, applied onto sterile bovine cortical bone slices, and then treated or left untreated with 30 mM NAC before 2-h incubation at 37 °C in a humidified chamber with 5 % CO_2. Cells adhered to bone slices were harvested at 12 h, 24 h, 48 h, and 96 h and then fixed with 4 % paraformaldehyde for 20 min before the immunofluorescence analysis. Photographs of representative images from each bone slice were taken using an inverted fluorescence microscope (SOPTOP®, Kuala Lumpur, Malaysia). Cell fusion was determined by merging images using the Image J software. Tests were performed in duplicate and repeated three times.

2.9. Immunofluorescence

U937 cells were subjected to centrifugal force or PEG-induced fusion, placed onto cortical bone slices, and treated or not with 30 mM NAC. After incubation for 12 h, 24 h, 48 h, and 96 h at 37 °C, these cells were fixed with 4 % paraformaldehyde for 30 min at 4 °C and treated with permeabilization buffer (80 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM $MgCl_2$, 50 mM NH_4Cl, and 1 % saponin). Blocking was performed by treating cells with 2 % bovine serum albumin (BSA) in PBS containing 50 mM NH_4Cl for 1 h at 37 °C. Permeabilized cells were incubated with primary antibodies (0.4 µg ml^{-1}) against CAII, CT, CTSK, V-ATPase, integrin $\beta 3$, DC-STAMP, MMP 9, ADAM12, or p-NF-$\kappa\beta$ in PBS with 1 % FCS at 37 °C for 60 min and then washed five times with PBS containing 50 mM NH_4Cl. Cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG secondary antibody (0.4 µg ml^{-1}, SC-2090), FITC-conjugated donkey anti-goat secondary antibody (0.4 µg ml^{-1}, SC-2024), or FITC-conjugated bovine anti-mouse IgG secondary antibody (0.4 µg ml^{-1}, SC-2366) for 30 min at 37 °C. Bone slices were mounted onto glass slides, covered with coverslips, and observed using an inverted fluorescence microscope (SOPTOP®, Kuala Lumpur, Malaysia). Recordings of fluorescent cells were made by observing successive fields along a W-shaped path through the bone slice. At least ten representative photographs were taken per bone slice. The fraction of immunoreactive cells were determined with the software ImageJ.

2.10. Assessment of actin rearrangements

Actin distribution in U937 cells was assessed following a published procedure [22] with some modifications. Briefly, U937 cells were subjected to centrifugal force or PEG-induced fusion before applying them to cortical bone slices. The cells were cultured for 96 h at 37 °C before two washes with RPMI. The cells were fixed with 4 % paraformaldehyde for 20 min at RT and permeabilized with acetic acid-methanol (50 : 50) at 4 °C for 24 h, followed by washing three times with PBS and blocking as indicated above. The cells were incubated with an mAb against actin (Abcam, ab8226, Cambridge, UK) and then blocked with 2 % BSA in PBS for 40 min at 37 °C before adding FITC-conjugated donkey anti-mouse secondary antibody for 30 min at 4 °C. Immunoreactive activity was examined with an inverted fluorescence microscope (SOPTOP®,

Kuala Lumpur, Malaysia). Each assay was performed in quadruplicate. U937 cells not subjected to centrifugal force or PEG-induced fusion and treated comparably with mAb against actin served as controls.

2.11. Statistical analysis

The experimental results were expressed as the mean ± standard deviation (SD) and were representative of the independent assays. Data were analyzed with two-way ANOVA or Student's *t*-test, and correlations between the variables assessed were evaluated with Tukey correlation tests.

3. Results

3.1. Centrifugal force induces TRAP and bone resorptive activities

TRAP is an enzyme expressed in osteoclasts that participates in bone resorption and is used as an OC marker. To determine cell viability changes and TRAP and bone resorptive activities of U937 cells, these were collected by centrifugation and then suspended repeatedly to obtain single-cell (*i.e.*, disaggregated cells) suspensions before seeding them onto bone slices. We attempted to determine the differential TRAP and bone resorptive activities of cells subjected separately to three rotor speeds corresponding to three centrifugal forces and pressures (2.4×10^{-6} g cm^{-2}, 2.4×10^{-4} g cm^{-2}, or 1.74×10^{-3} g cm^{-2}) applied for 24 h, 48 h, and 96 h. None of the centrifugal forces or exertion periods applied affected cell viability, when compared to the control cells that were not subjected to centrifugal force.

TRAP activity after 24 h of centrifugal force exertion at the three speeds assessed increased significantly when compared with control cells (unsubjected to centrifugal force). The increase of TRAP activity for the low, medium, and high speeds were 14 %, 21 %, and 29 % higher than control cell, respectively (**Figure 1A–C**). After 48 h, TRAP activity increased by 25 %, 25 %, and 32 % for each centrifugal force applied, respectively (Figure 1A–C). TRAP activity after 96 h of centrifugal exertion increased by 30 %, 35 %, and 38 % for each force level applied, respectively (Figure 1A–C). These results suggest that centrifugal force applied to single-cell suspensions sedimented on bone slices can induce expression of TRAP activity in a force and time-dependent manner.

To test for resorptive activity in cells subjected to centrifugal force, three different rotor speeds were used. Control cells without centrifugal exertion did not reveal changes in bovine cortical bone slices as their surfaces remained homogeneous, exhibiting normal bone appearance and without evidence of bone resorption. In the bovine cortical bone slices subjected to centrifugal force, different resorption degrees were observed after 24 h of centrifugal exertion. The percentages of bone resorption area were 5 %, 7 %, and 8 % for the low, medium, and high centrifugal levels, respectively (Figure 1D–F). Furthermore, bone slices showed a significant increase in resorption after 48 h of centrifugal force application. These percentages were 9 %, 12 %, and 14 % for the respective centrifugal forces applied (Figure 1D–F). After 96 h of centrifugal force application, the percentages of bone resorption were 12 %, 15 %, and 14 % for the respective centrifugal forces indicated above (Figure 1D–F).

Small, irregular bone resorption areas were observed on the bovine cortical bone slices for the three centrifugal forces applied; likewise, discrete visible bone resorption areas were identified, showing diameters close to the size of individual cells. Other bone slices showed rounded resorption areas with relatively larger diameters; it is possible that in these areas, an accumulation

of cells took place. Additionally, the most significant number of resorption areas was found in the center of the bone slices. Representative images of $TRAP^+$ cells and bone resorbed areas are shown (Figure 1G, H). The intermediate exertion level, corresponding to a centrifugal force P of 2.4×10^{-4} g cm^{-2} was the optimal resorption-eliciting condition since higher centrifugal forces did not induce higher bone resorptive activities. Considering this finding, all experiments were carried out at the medium centrifugal force for 96 h, except otherwise stated. These results suggest that bone resorption activity depended on both centrifugal force and time of its application but reached a limit after applying the medium centrifugal force tested.

To test the effects of redox state modification on TRAP and bone resorption activities, U937 cells, PBMCs, and PDL fibroblasts were placed on bone slices. Cells on bone slices were treated or left untreated with 30 mM NAC and subjected to medium centrifugal force (2.4×10^{-4} g cm^{-2}). U937 cells fused or not fused with PEG were cultured on bone slices without centrifugal exertion. The analysis of TRAP activity showed that in cells subjected to centrifugal

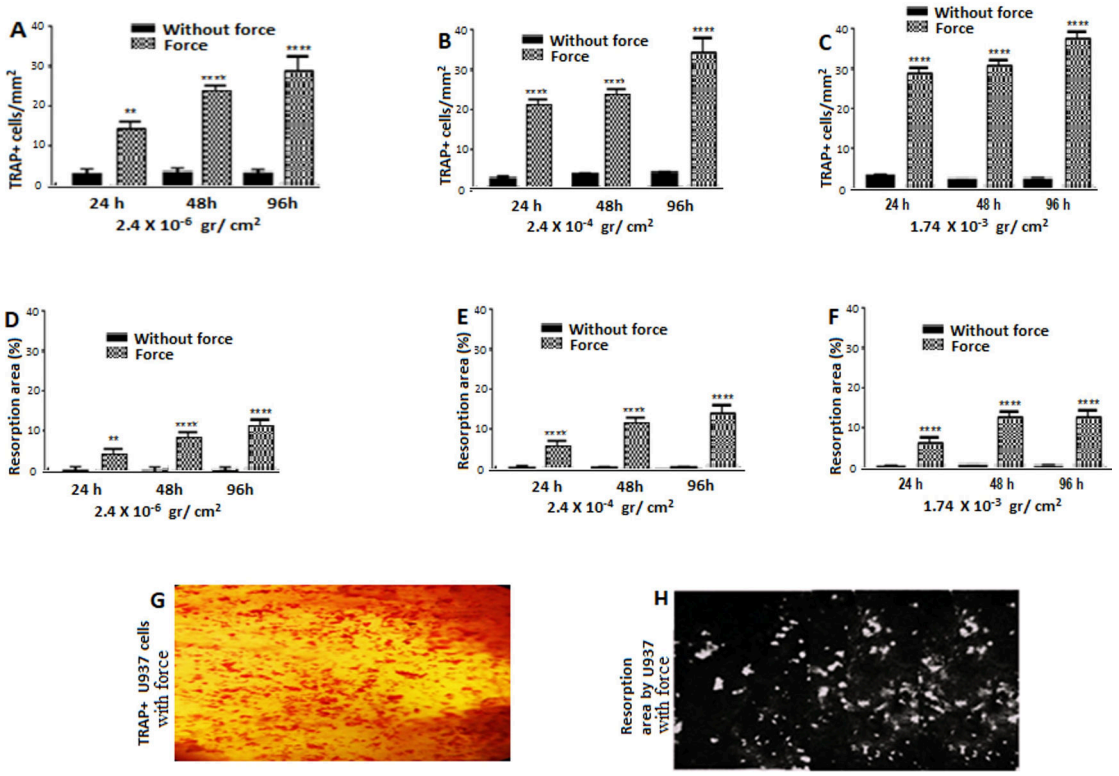

Figure 1. Induction of TRAP and bone resorption activities by centrifugal force. U937 cells (3×10^4 cells were separately placed on bone slices and subjected to centrifugal force at three different constant speeds during the indicated times at 37 °C. **A–C** Positive cells to TRAP activity per area unit found at increasing days of culture and centrifugal forces are shown. Cells without centrifugal force application were used as controls. **D–F** Bone resorption activity is shown as the percentage of resorbed area according days of culture and centrifugal force levels. **G** Representative image of cells with TRAP activity in bone slices. Arrows show $TRAP^+$ multinucleated cells. **H** Representative negative image showing areas of bone resorption activity. Arrows depict resorption pits. Results in A–F are shown as mean ± SD from three independent experiments performed in duplicate. Asterisks indicate statistically significant differences (** $p < 0.01$; *** $p < 0.001$) determined with two-way ANOVA between cells subjected to centrifugal force and those without centrifugal treatment. Photographs in **G** and **H** were taken from ten random fields of each well.

force, NAC treatment reduced their enzyme activity by 46 %, 24 %, and 18 % for U937 cells, PBMCs, and PDL fibroblasts, respectively, in comparison to cells subjected to centrifugal force without NAC treatment (**Figure 2A–C**).

On the other hand, U937 cells, PBMCs, and PDL fibroblasts that received neither centrifugal force nor NAC treatment showed only 3 %, 1.5 %, and 1.6 % of $TRAP^+$ cells, respectively (Figure 2A–C). PEG-fused U937 cells, PBMCs, and PDL fibroblasts without centrifugal force and NAC treatment showed 52 %, 24 %, and 36 % of $TRAP^+$ cells, respectively. When these cells were subjected to centrifugal force, in the absence of NAC treatment, their shares of $TRAP^+$ cells were 64 %, 29 %, and 29 %, respectively (Figure 2A–C). In the case of PEG-fused U937 cells, PBMCs, and PDL fibroblasts without application of centrifugal force, NAC treatment reduced their TRAP activity by 30 %, 17 %, and 23 %, respectively, in comparison to the PEG-fused cells subjected to centrifugal force in the absence of NAC treatment (Figure 2A–C).

Bone resorption activity for U937 cells, PBMCs, and PDL fibroblasts treated with NAC and subjected to centrifugal force was reduced by 10 %, 10.1 %, and 8.8 %, respectively, in comparison to cells of these three types subjected to centrifugal force only (Figure 2D–F). In PEG-fused U937 cells, PBMCs, and PDL fibroblasts not subjected to centrifugal force but given the NAC treatment, bone resorption activity was reduced by 27 %, 15 %, and 29 %, respectively, when compared to PEG-fused cells without NAC treatment (Figure 2D–F). Without NAC treatment, bone resorptive activities for PEG-fused U937 cells, PBMCs, and PDL fibroblasts were 35 %, 19 %, and 36 %, respectively. In contrast, centrifugal exertion, without NAC treatment, induced less bone resorptive activity than that observed for PEG-induced fusion, as the percentages for this activity were 13 %, 11 %, and 10 %, for the three cell types, respectively (Figure 2D–F). Images of $TRAP^+$ cells and bone resorbed areas are shown for U937 cells, PBMCs, and PDL fibroblasts treated or not with NAC compared to PEG-fused U937 cells (Figure 2G, H). These results revealed that both TRAP and bone resorptive activities induced by centrifugal force or PEG-induced fusion were significantly inhibited by antioxidant treatment.

3.2. DiO and DiD facilitate identification of PEG-induced fusion

DiO and DiD dyes belong to a family of lipophilic fluorescent stains for labeling cell membranes and other hydrophobic structures. To assess U937 cell fusions, these cells were separately treated with DiO and DiD and then mixed before fusing them with PEG or subjecting them to the centrifugal force in the absence or presence of NAC. The cells seeded onto bovine cortical bone slices were cultured for 24 h, 48 h, and 96 h. Cells treated with DiO and DiD without PEG-induced fusion, submission to centrifugal force, and NAC treatment were used as a control. Regardless of NAC treatment, images revealed no cell fusions (**Figure 3A–C**). The percentage of merged cells under centrifugal force were 9 % at 24 h, 12 % at 48 h, and 37 % at 96 h (Figure 3D). However, the fusion events observed under centrifugal force decreased in comparison to those observed in PEG-fused cells. The cells treated with NAC did not show significant changes according to a Student's t-test as compared to cells submitted to centrifugal force and without NAC treatment ($p > 0.05$) (Figure 3A–C).

In PEG-fused cells, a merge of images of about 40 % was observed at 24 h of culture. In addition, cell bodies were larger than those of the control group (Figure 3A–D). The percentage of multinucleated cells revealing merges was 51.25 % at 48 h of culture. This was statistically significant when compared to cells without PEG-induced fusion, centrifugal force application, and NAC treatment (Figure 3B, D). The merging share increased by 64.74 % at 96 h of culture, and cell size increased compared to cells without NAC treatment (Figure 3C, D).

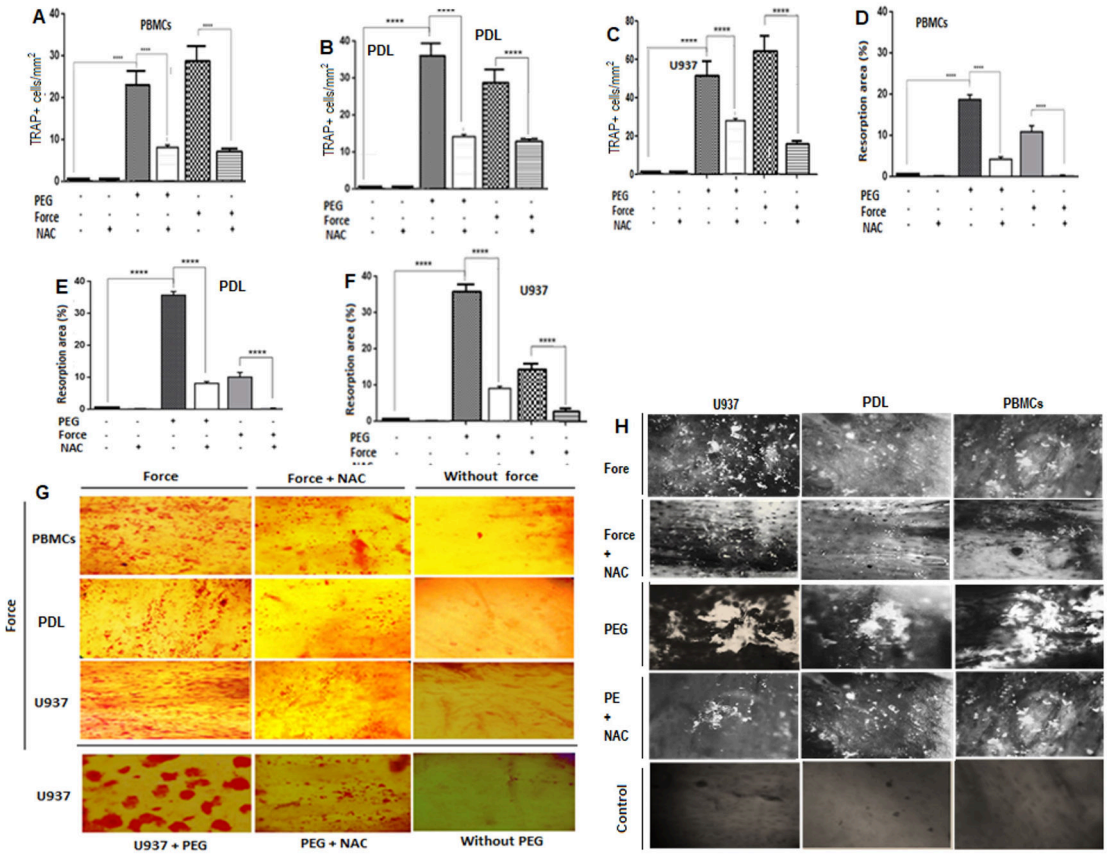

Figure 2. Redox environment effect on TRAP and bone resorption activities induced by centrifugal forces. U937 cells, PBMCs, and PDL fibroblasts with and without PEG-induced fusion were placed on bone slices and cells treated, or left untreated, with NAC (30 mM). Bone slices were subjected to a centrifugal force equivalent to 2.4×10^{-4} g cm^{-2} for 96 h at 37 °C. Control cells without PEG-induced fusion were cultured under the same conditions without being subjected to centrifugal force but treated with NAC. Bone slices were assayed for TRAP and bone resorption activities. **A–C** Percentages of positive cells to TRAP activity per area unit in bone slices; treatments are indicated below each panel. **D–F** Bone resorption activity is shown as the percentage of total area reabsorbed in bone slices; treatments are indicated below each panel. Asterisks indicate statistically significant differences (*** $p < 0.001$) between cells subjected to centrifugal force and the indicated control cells. **G** Images showing positive cells to TRAP activity in bone slices that were incubated with the indicated cells and subjected to centrifugal force. PEG-induced fusion or NAC treatment are also shown. Arrows show TRAP$^+$ multinucleated cells. **H** Negative images revealing areas of bone resorption activity for the indicated cells that were subjected to centrifugal force, PEG-induced fusion or NAC treatment are also shown. Arrows show resorption pits.

In the case of PEG-fused cells treated with NAC, the percentages of multinucleated cells showing merges were 22 %, 47.5 %, and 50 % at 24 h, 48 h, and 96 h of culture, respectively. A reduction in image merging size was observed compared to the control consisting of PEG-fused cells without NAC treatment (Figure 3A–D).

3.3. U937 cells subjected to centrifugal force increased their ROS levels

To evaluate U937 ROS production, cells were seeded onto bovine cortical bone slices, treated with DHE, and cultured for 12 h, 24 h, 48 h, and 96 h. Control cells cultured without any treatment showed very low or negligible fluorescence signal intensity. Similar fluorescence signals were observed in the cells treated with 30 mM NAC. In the case of cells subjected to centrifugal force, an increase in median fluorescence intensity (MFI) was observed between 24 h and 96 h

of culture when compared to the control cells without any treatment. However, the fluorescence signals of the cells subjected to centrifugal force were as intense as those observed in the PEG-fused cells, except at 96 h when the cells subjected to centrifugal force showed an MFI greater than of PEG-fused cells (**Figure 4A–E**). In the case of cells subjected to centrifugal force and NAC treatment, their MFI was lower than that of cells without NAC treatment. These results revealed that cells subjected to centrifugal force increased the intracellular concentration of ROS and that NAC antioxidant activity suppressed this effect.

The PEG-induced fusion of U937 cells increased the MFI after 24 h of culture compared to control cells unsubjected to centrifugal force and treatments with PEG and NAC (Figure 4A–E). However, this MFI decreased between 48 h and 96 h of culture, except that it remained significantly higher ($p < 0.001$) than that observed in control cells. In the case of PEG-fused and NAC-treated cells,

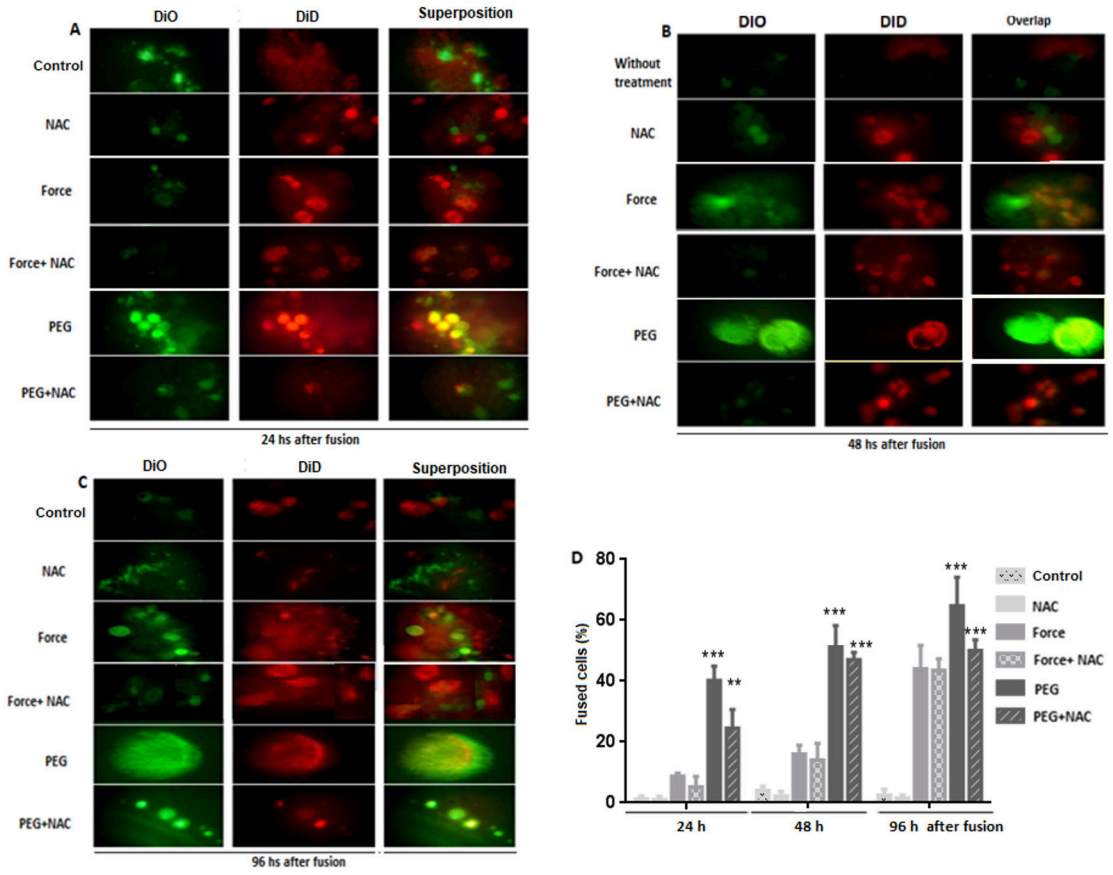

Figure 3. Staining of U937 cells onto bovine cortical bone slices with lipophilic DiO and DiD dyes. U937 cells (3×10^4) previously incubated with DiO or DiD were subjected or not to PEG-induced fusion and then placed onto bovine cortical bone slices. Each bone slice with the attached cells was treated or not with NAC (30 mM). Bone slices were subjected to a centrifugal force equivalent to a pressure of 2.3×10^{-4} g cm^{-2}. Cells incubated with DiD and DiO and without PEG-induced fusion, centrifugal force application, and NAC treatment were used as a control. Cells were fixed and analyzed for the percentage of cell fusion in terms of the image merging of cells stained with DiD and DiO. **A–C** Photographs of cells treated with DiD or DiO and immediately submitted to centrifugal force, PEG-induce fusion, or NAC treatment for 24 h, 48 h or 96 h. Cells incubated with DiD and DiO and subjected to centrifugal force before being fused with PEG or treated with NAC. U937 cells without centrifugal force stress, PEG-induced fusion, and NAC treatment were used as a control. **D** Quantification of fused cells (%) according to the image merging of cells stained with DiD and DiO. Data are presented as mean ± SD from three independent experiments performed in duplicate. Asterisks indicate statistically significant differences (** $p < 0.01$; *** $p < 0.001$).

the MFI was lower than that observed in control cells only fused with PEG and not treated with NAC (Figure 4A–E). These results suggest that regardless of the centrifugal exertion, PEG-induced fusion induced osteoclast-like activities, including oxidative stress.

3.4. Centrifugal force induces the formation of actin rings in U937 cells

Actin cytoskeleton in osteoclasts is necessary for bone resorption through actin rings formation and the development of a V-ATPase-rich ruffled plasma membrane. We assessed the centrifugal force effect on actin ring formation in U937 cells using a mAb against actin and FITC-conjugated secondary antibody. Control cells without PEG-induced fusion, centrifugal force, and NAC treatment, showed an individualized and homogeneous fluorescence pattern. The same situation emerged in control cells treated with NAC (**Figure 5A, B**). The cells subjected to centrifugal

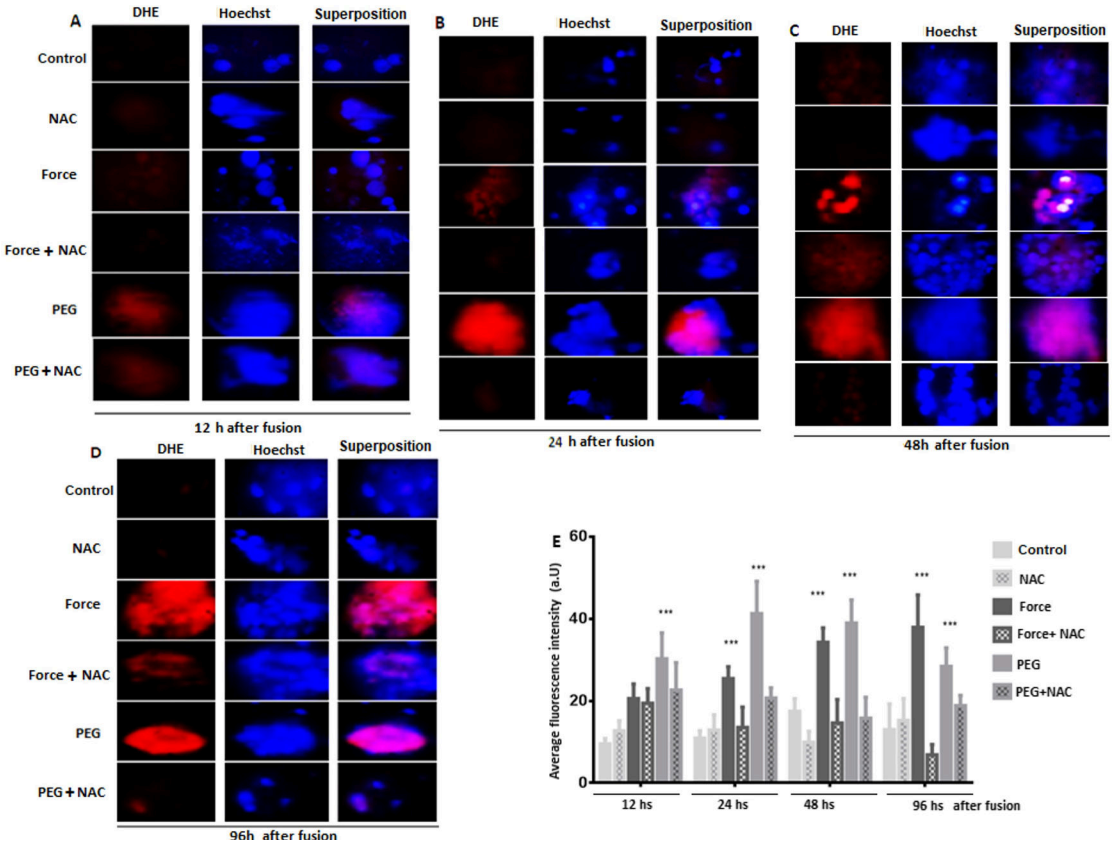

Figure 4. Assessment of ROS activity in U937 cells subjected to centrifugal force and treated or not with PEG or NAC. U937 cells (3×10^4) previously subjected or not subjected to PEG-induced fusion were placed on bone slices. Each bone slice with the attached cells was treated or not with NAC (30 mM). Bone slices were subjected to a centrifugal force equivalent to a pressure of 2.4×10^{-4} g cm^{-2} for 12 h, 24 h, 48 h, or 96 h at 37 °C. Control cells without PEG-induced fusion were cultured under the same conditions without being subjected to centrifugal force and NAC treatment. Cells attached to bone slices were treated with dihydroethidium (DHE), fixed, and treated with Hoechst. MFI was quantified using the ImageJ 1.3 software. **A–D** Representative images of cells subjected to centrifugal force, PEG-induced fusion, or NAC treatment are shown. The control consisted of cells without centrifugal force application, PEG-induced fusion, and NAC treatment. **E** MFI (arbitrary units) is shown for cells submitted to the treatments described in **A–D**. Data are shown as mean ± SD, and asterisks indicate statistically significant differences (*** $p < 0.001$).

force for 96 h showed peripherally distributed fluorescence in 50 % of the cells. However, some cells showed peripheral nuclei, suggesting that these cells clumped together when placed onto bone slices, facilitating cell fusion during centrifugal force exertion (Figure 5A).

The cells subjected to centrifugal force and treated with NAC showed fluorescence signals distributed peripherally in 8 % of the cell population and remainder showed a homogeneous fluorescence pattern (Figs. 5A, B). In PEG-fused cells, large ring-shaped peripheral fluorescent structures appeared in 100 % of the cells analyzed. DAPI staining revealed nuclei located on the periphery of cells (Figure 5A). In the case of cells fused with PEG and treated with NAC, the cells showing typical actin rings were reduced to 24 % as compared to control PEG-fused cells without NAC treatment, while the remaining cells (76 %) showed the shape and fluorescence signal distribution similar to those observed in control cells. However, NAC-treated cells had a diameter larger than that of control cells.

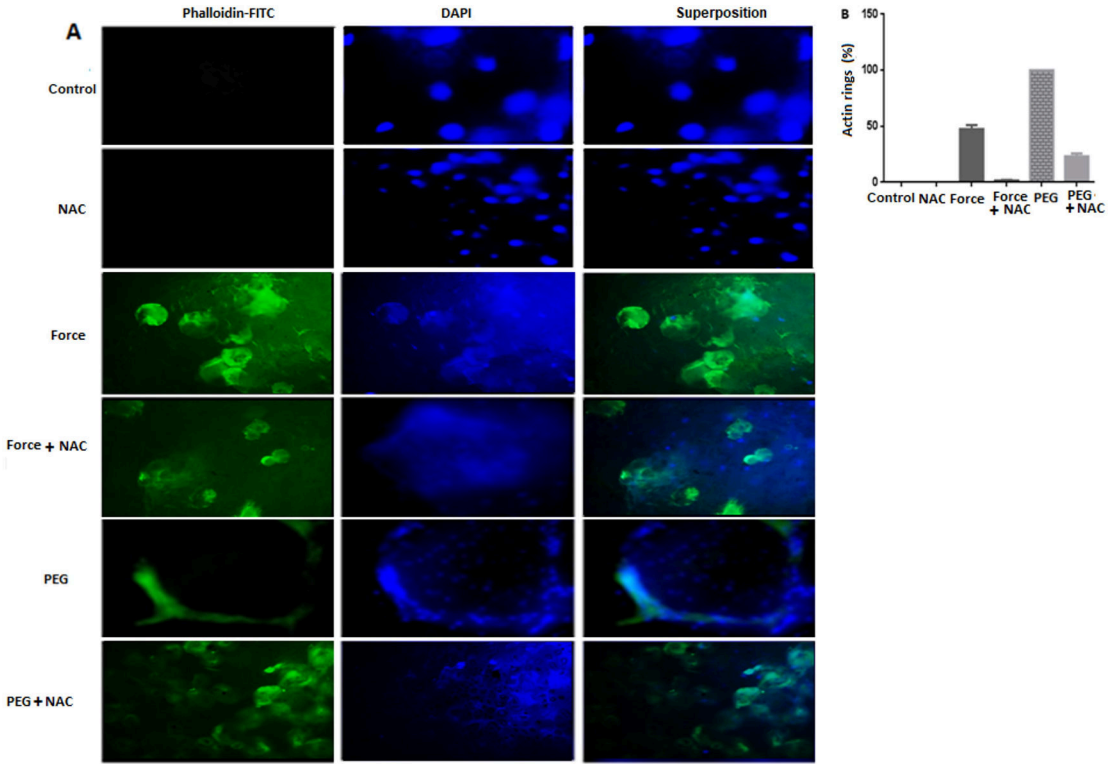

Figure 5. Assessment of actin rings from U937 cells subjected to centrifugal force and treated with PEG or NAC. U937 cells (3×10^4) previously subjected or unsubjected to PEG-induced fusion were placed on bone slices. Each bone slice with the attached cells was treated or not with NAC (30 mM). Bone slices were subjected to a centrifugal force equivalent to a pressure of 2.4×10^{-4} g cm^{-2} for 96 h at 37 °C. Control cells without PEG-induced fusion were cultured under the same conditions without being subjected to centrifugal exertion and NAC treatment. Cells were fixed and permeabilized. Cells were incubated with either monoclonal antibodies against actin or phalloidin and then stained with DAPI. **A** Cells subjected to centrifugal force, PEG-induced fusion, or NAC treatment and controls; arrows show peripheral nuclei. **B** Percentage of cells positive for actin ring formation in the cells treated as indicated in **A**.

3.5. OC differentiation markers are induced by centrifugal force or PEG-induced fusion

To assess the characteristic osteoclast differentiation markers, U937 cells subjected to pressure-generating centrifugal force (2.4×10^{-4} g cm^{-2}) or PEG-induced fusion were cultured on bovine cortical bone slices for 48 or 96 h. The expression of differentiation markers was evidenced with antibodies directed to CAII, CT, CTSK, V-ATPase, integrin $\beta 3$, DC-STAMP, MMP-9, ADAM 12, and p-NF-$\kappa\beta$. Images of p-NF-$\kappa\beta$-positive cells are shown (**Figure 6A**). Fluorescence signals for p-NF-$\kappa\beta$ were absent in cells without PEG-induced fusion, centrifugal force, and NAC treatment when the detection assays were made at 48 h and 96 h of culture. A similar result was observed in cells treated with 30 mM NAC or in cells incubated with isotype control antibody (data not shown). In cells subjected to centrifugal force or PEG-induced fusion, the fluorescence signals for CAII, CT, CTSK, V-ATPase, integrin $\beta 3$, DC-STAMP, MMP-9, ADAM 12, and p-NF-$\kappa\beta$ were positive at 48 h and 96 h of culture. These fluorescence signals varied depending on the protein analyzed. At the same time, they decreased when the PEG-fused cells were treated with 30 mM NAC (Figure 6B–J).

4. Discussion

Mechanical stress is an essential factor in bone homeostasis, maintained by balancing bone resorption by OCs and bone formation by OBs. Mechanical forces cause deformation of the extracellular matrix, which contains mechanoreceptor molecules activated by applying different forces. These molecules can detect and convert microenvironmental biomechanical stimuli into intracellular biochemical signals. Moreover, it has been speculated that nuclear deformation drives chromatin configuration changes, leading to gene expression variation. Previous studies have examined osteoblast, fibroblast, and osteoclast responses to various types of mechanical stimulation. Mechanical stimuli, including the tensile force [9], compression force, fluid flow [11], hydrostatic pressure, tension associated with rotation, and other stimuli, are critical bone remodeling factors. The induction of osteoclastogenesis by mechanical compressive force has been previously documented [4]. Application of orthodontic forces to teeth leads to bone resorption and formation, whereas the compressed PDL cells release osteoclast inductive molecules resulting in alveolar bone resorption.

In the present study, PBMCs, PDL fibroblasts, and U937 cells cultures under 96 h of mechanical exertion increased significantly TRAP activity and resorption areas in bone slices compared with control cultures without PEG-induced fusion, centrifugal force, and NAC treatment. Interestingly, bone resorption decreased significantly when NAC treatment was used. These results suggest that cells subjected to centrifugal force respond by activating a signaling pathway that induces TRAP activity. However, the total resorption area and its depth observed in bone slices with cells subjected to centrifugal force were lower than those observed for the PEG-fused cells. In the case of cells subjected to centrifugal force, the heterogeneity in sizes and shapes observed in the resorption areas could be interpreted as a consequence of the centrifugal force leading to either cell aggregation or cell fusion, thereby improving bone resorption activity. Non-fused mononuclear cells subjected to centrifugal force express activities and characteristics previously considered exclusive to multinucleated osteoclasts.

Studies conducted by others showed the contrasting effects of different forces applied to cells, particularly in the presence of cell-differentiation factors such as RANKL. For instance, TRAP and bone resorption activities have increased and decreased depending on the exertion applied. Other studies have shown that microgravity promotes cell fusion without increasing osteoclast

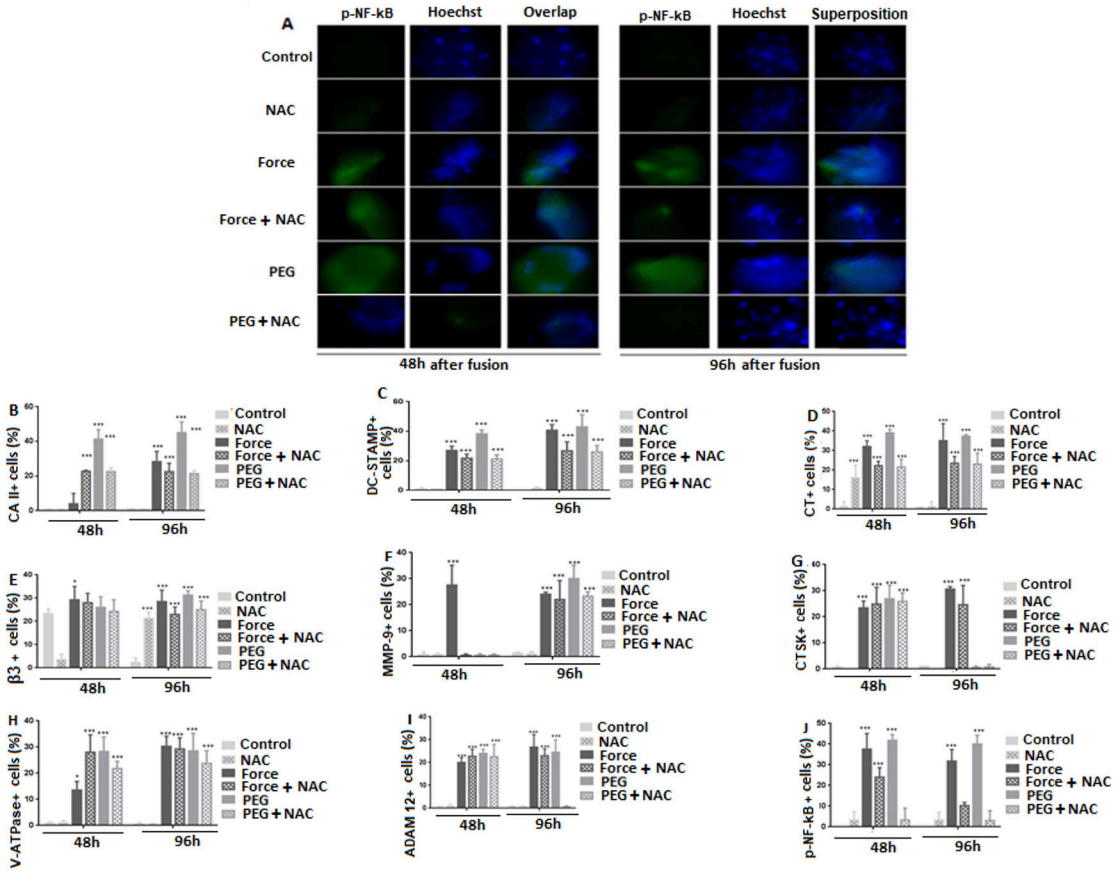

Figure 6. Expression of osteoclast differentiation markers in U937 cells subjected to centrifugal force and treated with PEG or NAC. U937 cells (3×10^4) previously subjected to PEG-induced fusion were placed on bone slices. Each bone slice with the attached cells was treated or not with NAC (30 mM). Bone slices were subjected to a centrifugal force equivalent to a pressure of 2.4×10^{-4} g cm^{-2} for 96 h at 37 °C. Control cells without PEG-induced fusion were cultured in the same conditions without being subjected to centrifugal force and NAC treatment. Cells were fixed and permeabilized before incubation with antibodies to CAII, CT, CTSK, V-ATPase, integrin β3, DC-STAMP, MMP-9, ADAM 12, and p-NF-$\kappa\beta$. **A** Cells positive to p-NF-$\kappa\beta$ at two indicated culture times; cell treatments with centrifugal force, PEG, or NAC are also shown. **B–J** Percentage of cells positive to nine indicated antibodies. Data are shown as mean percentages ± SD obtained from three independent experiments conducted in duplicate. Asterisks indicate statistically significant differences (* $p < 0.05$; *** $p < 0.001$) determined using a Student's t-test.

differentiation but by increasing resorption activity. Similarly, compression on RAW264.7 cells results in multinucleated cells, suggesting that compression forces accelerate cell fusion [24]. Lastly, mechanical stress directly suppresses osteoclast differentiation, suggesting a delayed differentiation in RAW264.7 cells.

Furthermore, applications of centrifugal force to co-cultures of monocytes with PDL or gingival fibroblasts inhibited osteoclastogenesis by promoting osteoprotegerin (OPG) production since OPG binds to RANKL and inhibits osteoclast development [25]. In the present study, we showed that PBMCs, PDL fibroblasts, and U937 cells, seeded on bovine cortical bone slices and subjected to centrifugal, experienced bone resorption activity without adding differentiation factors or performing co-cultures. These findings contribute to understanding the effect of force stress on bone resorption.

Our results show that centrifugal force or PEG-induced cell fusion increases via oxidative stress by generating ROS. Likewise, exposure of PDL fibroblasts to mechanical compression increases intracellular ROS production [26]. Our results support the idea that the oxidative stress in cultured cells, subjected to centrifugal force or PEG-induced fusion, triggers biochemical pathways leading to TRAP and bone resorption activities characteristic of osteoclasts. Our results also confirm that oxidative stress can activate the NF-$\kappa\beta$ pathway [27].

NAC treatment of PEG-fused cells reduced the percentage of multinucleated cells showing merged images when stained with DiD and DiO. Pictures were taken with an inverted microscope directly onto bovine cortical bone slices, but clarity was compromised as they would be on a glass slide. These NAC-treated cells decreased their sizes compared to PEG-fused control cells without NAC treatment, suggesting that the decrease in intracellular oxidative activity interferes with the progression of PEG-induced cell fusion, which could explain the drop in TRAP and bone resorption activities observed in cells treated with NAC. A share of 44 % merged cell images occurred only in cultures subjected to centrifugal force for 96 h. These images showed cell sizes smaller than those of PEG-fused cells. Crowded cells were also visible, yet smaller than those of PEG-fused cells. The smaller-sized cells persisted despite repeated resuspension before seeding them onto bone slices. This phenomenon suggests that these cells tend to form clusters or that the stress generated by centrifugal force induces them to fuse. The control cells, not-subjected to centrifugal force, remained scattered on the bone slice surface. In our hands, the distinction between cell aggregation and polykarion formation due to centrifugal exertion depended on the ability to suspend the cells efficiently before placing them on coverslips.

Cultures subjected to centrifugal force revealed some actin ring-positive cells. These oval or circular actin signals were not similar in size or fluorescence intensity to those observed in PEG-fused cells. Since the cells were seeded on bone slices, only conventional fluorescence microscopy allowed their observation, but it hindered the differentiation of actin ring structures by epifluorescence. Some cells had their nuclei located close to the plasma membrane, suggesting that fusion could occur due to cell crowding on bone slices or stress generated by force-induced cell fusion. In NAC-treated cells, actin was evenly present throughout cells, with only 8 % of it seen on the cell periphery.

On the other hand, all PEG-fused cells showed actin ring-like structures and revealed nuclei close to the plasma membrane, suggesting that PEG-induced cell fusion triggers changes in the redistribution of the actin cytoskeleton. PEG-fused and NAC-treated cells showed few cells with actin ring-like structures, while most cells were similar to control cells in terms of fluorescence shape and distribution and absence of actin ring-like structures, suggesting that antioxidant activity affects the formation of actin rings. Therefore, these results imply that antioxidant activity affects actin ring formation. These data support the idea that oxidative stress generated in U937 cells by centrifugal force or cell membrane fusion induced by PEG triggers the activation of pathways required to build actin ring-like structures, expression of TRAP, and bone resorption, all of which osteoclast characteristics.

In contrast to our study, other studies addressing biochemical and morphological changes induced in precursors of osteoclast-like cells by mechanical compressive forces have used differentiation factors such as hormones, cytokines, dexamethasone, vitamin D3, M-CSF, and RANKL instead of PEG-induced fusion of macrophages. However, our results further confirm that mechanical forces induce the expression of osteoclastic markers and ROS production, except that our study adds insights into the involvement of ROS in osteoclastic activity.

Centrifugal force to study mechanical stress effects allows for strength control while keeping cell culture conditions constant [28]. One of the disadvantages of centrifugal force for studying osteoclastic activity in cell cultures is that cells are subjected to radial forces and are in fast-moving conditions instead of being under a static status [4]. Finally, we identified osteoclast-specific markers such as CAII, CT, CstK, V-ATPase, integrin $\beta 3$, DC-STAMP, MMP-9, ADAM-12, and p-NF-$\kappa\beta$ in U937 cells subjected to centrifugal force or fused with PEG. DC- and OC-STAMP expression in RAW264.7 cells subjected to continuous compressive forces is well known [6]. On the other hand, the fluorescent signal intensity for these markers decreased significantly or was not detected in NAC-treated cells, as observed in control cells without PEG-induced fusion, centrifugal force, and NAC treatment. These results support the hypothesis that oxidative stress generated during centrifugal force or PEG-induced fusion of cell membranes can induce osteoclast-specific cytoplasmic markers.

5. Conclusions

In summary, we have shown that U937 cells subjected to centrifugal force or PEG-induced fusion respond by increasing intracellular levels of ROS, forming actin-like rings, and expressing TRAP and bone resorption activities, in addition to expressing typical osteoclast markers. However, these specific osteoclast events were significantly decreased by NAC treatment, revealing that an increased intracellular oxidative activity in both experimental models tested is at least associated with or implicated in the differentiation of osteoclast-like cells. Furthermore, we highlight that we employed a bone resorption model without involving conventional osteoclast differentiation factors such as RANKL or co-cultures before the use of mechanical stress.

The results of this work provide evidence that oxidative stress and cell fusion are inducers of osteoclast-like features. On the other hand, the results also provide new insights into the molecular mechanisms associated with mechanical force-induced osteoclastogenesis that could guide research on alveolar bone resorption and restoration during orthodontic interventions.

Finally, our results suggest that centrifugal force applied to single-cell suspensions can induce TRAP activity expression according to intensity and exertion time. Also, we have consistently observed that bone resorption depended on both centrifugal exertion and the time of its application. Both activities are fully compatible with osteoclast differentiation theory. Our most striking finding was that centrifugal force concomitantly induced ROS generation, suppressed by treatment with the antioxidant agent NAC. Overall, oxidative stress is concomitant with TRAP and bone resorption activities during osteoclastic differentiation.

6. Abbreviations

OCs: osteoclasts. ROS: reactive oxygen species. PBMCs: peripheral blood mononuclear cells. PDL: periodontal ligament fibroblasts. PEG: polyethylene glycol. NAC: N-acetylcysteine. TRAP: tartrate-resistant acid phosphatase. ECM: extracellular matrix. OC-STAMP: Osteoclast stimulatory transmembrane protein. DC-STAMP: Dendritic Cell-Specific Transmembrane Protein. NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1. RANKL: nuclear factor-$\kappa\beta$ ligand. RANK: receptor activator of nuclear factor-kappa B. M-CSF: macrophage colony-stimulating factor. OBs: osteoblasts. OTM: orthodontic tooth movement. BMMCs: bone marrow mononuclear cells. FCS: fetal calf serum. DHE: dihydroethidium. DiO: 3,3-Dioctadecyloxacarbocyanine. DiD: 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine. CAII: carbonic anhydrase II. CT: calcitonin receptor. CTSK: cathepsin K. V-ATPase: Vacuolar proton-translocating ATPases. MMP

9: Matrix metallopeptidase 9. ADAM12: Disintegrin and metalloproteinase domain-containing protein 12. p-NF-$\kappa\beta$: nuclear factor-kappa B. FITC: fluorescein isothiocyanate. MFI: median fluorescence intensity.

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Not applicable.

8. Author contributions

P. Moreno, B. Pazos, J. S. Bedoya, A. Bedoya-Rodriguez conducted experiments, analyzed and interpretated data, and write this paper's draft. C.A. Guerrero contributed to the conception and design of this study, as well as to analysis and interpretation of data. O. Acosta supported data analysis and critical writing of this manuscript.

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10. Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Actividad osteoclástica de células U937, células mononucleares de sangre periférica y fibroblastos de ligamento periodontal sujetos a estrés mecánico por fuerza centrifuga

Resumen: Algunos modelos *in vitro* han investigado las consecuencias de los estímulos mecánicos en osteoclastos (OC). Sin embargo, los mecanismos por los cuales las fuerzas mecánicas detonan las respuestas osteoclásticas aun no se entienden bien, y la generación de especies reactivas de oxígeno (ROS) y su relación con la resorción ósea en OCs bajo la influencia de fuerzas mecánicas requiere de investigación. El presente trabajo examinó el papel de la aplicación de fuerza centrífuga en la producción de ROS y su efecto en la actividad osteoclástica y la diferenciación. Se sometieron a fuerza centrífuga macrófagos humanos U937, células mononucleares de sangre periférica (PBMCs) y fibroblastos del ligamento periodontal tratados con polietilenglicol (PEG) o N-acetilcisteína (NAC). Se midieron marcadores osteoclásticos como fosfatasa ácida tartrato-resistente (TRAP) y actividades de resorción ósea. Se determinaron los niveles de ROS y la formación de anillos de actina. Se estudiaron también las repuestas de las células U937 a la fuerza centrífuga y a fusión inducida por PEG. Las células individuales sometidas a fuerza centrífuga aumentaron los niveles de ROS formaron anillos similares a anillos de actina, revelaron actividades de expresión de TRAP y de resorción ósea, y expresaron marcadores osteoclásticos típicos. Las células U937 control fusionadas con PEG también mostraron estos efectos, y el tratamiento celular con NAC detuvo todas estas respuestas. La fuerza centrífuga, así como la fusión celular inducida por PEG, pueden promover características osteoclásticas, incluyendo estrés oxidativo. El presente modelo experimental nos permite entender los mecanismos subyacentes a la diferenciación osteoclástica asociada con la producción de ROS estimulada por fuerzas compresivas mecánicas, donde la NAC puede contribuir a reducir la condición de estrés oxidativo.

Palabras Clave: resorción ósea; fuerza centrífuga; fusión monocítica, ligamento periodontal; actividad osteoclástica; polietilenglicol.

Atividade similar a osteoblastos de células U937, células mononucleares do sangue periféricos e fibroblastos do ligamento periodontal submetidas a estresse mecânico por força centrífuga

Resumo: Vários modelos *in vitro* têm investigado as consequências dos estímulos mecânicos nos osteoclastos (OCs). No entanto, os mecanismos pelos quais as forças mecânicas desencadeiam as respostas dos OCs permanecem mal compreendidos. Adicionalmente, a geração de espécies reativas de oxigênio (ROS) e sua relação com a reabsorção óssea em OCs sob a influência de forças mecânicas requerem investigação. O presente trabalho estudou o papel da aplicação da força centrífuga na produção de ROS e seu efeito sobre a atividade e diferenciação dos OCs. Macrófagos humanos U937, células mononucleares do sangue periférico (PBMCs) e fibroblastos do ligamento periodontal (PDL) tratados com polietileno glicol (PEG) ou N-acetilcisteína (NAC) foram sujeitos à força centrífuga. Medimos marcadores de OCs como fosfatase ácida resistente a tartarato (TRAP) e a atividade de reabsorção óssea. Determinamos os níveis de ROS e a formação de anéis de actina. Além disso, estudamos a resposta das células U937 à força centrífuga e à fusão induzida pelo PEG. Células individuais submetidas ao esforço centrífugo aumentaram seus níveis de ROS, formaram anéis semelhantes à actina, mostraram expressão TRAP e reabsorção óssea e expressaram marcadores típicos de OCs. As células U937 controle fusionadas com PEG também mostraram esses efeitos, e o tratamento celular com NAC parou todas as respostas. A força centrífuga, bem como a fusão celular induzida pelo PEG, pode promover características semelhantes às dos OCs, incluindo estresse oxidativo. O presente modelo experimental nos permitiu compreender os mecanismos subjacentes à diferenciação de OCs associada à produção de ROS estimulada pela força compressiva mecânica, onde o NAC pode contribuir a reduzir a condição de estresse oxidativo.

Palavras-chave: reabsorção óssea; Força centrífuga; Fusão de monócitos; Ligamento periodontal; Atividade de osteoclastos; Polietileno glicol.

Pedro Moreno Beltran is a dentist, MSc. in biochemistry from the National university of Colombia. Currently, he is an associate teacher of international polytechnic Bogotá Colombia. His fields of interest are biochemistry applied to dental sciences, especially the role of bone metabolism in dental and periodontal homeostasis.

ORCID: 0000-0002-8477-9220

Angie Andrea Bedoya Rodriguez is a dentist and a MSc in biochemistry. She is currently a cPhD in biotechnology at the National University of Colombia. She belongs to the virus molecular biology research group of the Faculty of Medicine of the National University of Colombia.

ORCID: 0000-0003-2293-6128

Bibiana Pazos Valencia is a dentist from Valle University and a specialist in orthodontics and maxillary orthopedics from the National University of Colombia. Exclusive private practice in orthodontics and maxillary orthopedics since 2013 in Cali Colombia.

ORCID: 0000-0002-8659-9047

Juan Sebastián Bedoya Trujillo is a dentist from Antioquia University, Orthodontics and maxillary orthopedics specialist from National University of Colombia, Exclusive private practice in orthodontics and maxillary orthopedics since 2015, professor of American´s vision university foundation at Medellín and research advisor.

ORCID: 0000-0002-4727-1946

Orlando Acosta is a chemist and biologist who holds an MSc. in biochemistry and a PhD. in molecular virology. His research activity has included basic studies on bone metabolism and plant and animal viruses. In the field of genetically modified organisms, he has participated in studies on the production of herbicide-resistant transgenic plants. He coordinates the virus molecular biology group and is an emeritus researcher at Minciencias.

ORCID: 0000-0002-1267-098X

Carlos Arturo Guerrero Fonseca is a medical who holds an MSc. in Pharmacology, MSc. in Genetics and PhD. in biochemistry. His research activity has included basic studies on bone metabolism, animal viruses and oncolytic virus. He coordinates the master's degree in

biochemistry and the laboratory of molecular biology of viruses of the medical school of the national university and is an emeritus researcher at Minciencias.

ORCID: 0000-0003-2863-6345