Histol Histopathol (2013) 28: 1185-1196
DOI: 10.14670/HH-28.1185

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

RANKL is downregulated in bone cells by physical activity (treadmill and vibration stimulation training) in rat with glucocorticoid-induced osteoporosis

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Summary. The aim of this study was to investigate bone tissue and plasma levels of RANKL and OPG in rats with prednisolone-induced osteoporosis and to evaluate the outcomes of physical activity on the skeletal system by treadmill and vibration platform training. Osteoporosis is a disease characterised by low bone mass and structural deterioration of bone tissue leading to bone fragility. Vibration exercise is a new and effective measure to prevent muscular atrophy and osteoporosis.

The animals were divided into 5 groups. 1: control rats; 2: rats with osteoporosis receiving prednisolone; 3: rats receiving prednisolone and treadmill training; 4: rats receiving prednisolone and vibration stimulation training; 5: rats receiving prednisolone, treadmill and vibration stimulation training. For bone evaluations we used whole-body scans, histology and histomorphometric analysis. RANKL and OPG expression was evaluated by immunohistochemistry and biochemical analysis.

After treatment, our data demonstrated that RANKL expression was significantly increased in groups 2 and 3 and decreased in groups 4 and 5. Conversely, OPG expression was significantly decreased in groups 2 and 3 and increased in groups 4 and 5.

In conclusion, our findings suggest that mechanical stimulation inhibits the activity of RANKL. This finding provides new insights into the occurrence and progression of osteoporosis.

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Key words: Bone, Osteoporosis, Glucocorticoids, RANKL, OPG

Introduction

Vibration is a mechanical stimulus characterized by a repetitive motion back and forth in the same pattern. When an athlete stands on a vibrating platform, the repetitive movement presents a strong stimulus to musculoskeletal structure, resulting in changes in muscle stiffness in response to the vibration, as physiological adaptations to accommodate the vibratory waves (Cardinale and Bosco, 2003). Vibration exercise is a new and effective measure to prevent muscular atrophy and osteoporosis (Rittweger et al., 2002; Torvinen et al., 2002; Roelants et al., 2004; Verschueren et al., 2004). Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue leading to bone fragility and an increased risk of hip, spine, and wrist fractures (Gold, 2001; Tosteson and Hammond, 2002; Lips and van Schoor, 2005). The latter could be associated with osteoarthritis leading to several histopathological changes and determining an endogenous cellular response (Musumeci et al., 2011a-c, 2012a,b). Annually, in the United States, about 1.5 million osteoporosis-related fractures occur, and there is expected to be an increase of 50 % by 2025 (Adachi et al., 2003). Three common risk factors for osteoporosis are age, immobility and low body weight, specially in postmenopausal women. Prevention has been suggested as an important approach to reduce the incidence of osteoporosis. Physical activity increases energy

metabolism, contributes to a healthy energy balance and can be used to increase lean mass and bone mass. Physical activity is also beneficial in attenuating or improving chronic disease conditions (Burge et al., 2007; Loreto et al., 2011a). To reach these goals, persons with diabetes and elderly people use physical exercise regimes to reduce the risk of osteoporosis and fractures (Catenacci and Wyatt, 2007; Rizzoli et al., 2009). However, immobility, age, and other frailties may prevent optimal participation in exercise regimes designed for osteoporosis patients. Reports indicate that a mechanical stimulus in the form of vibration, traveling from the sole of the foot through the skeleton, is anabolic for bone (Rubin et al., 2001a,b, 2002; Kenny et al., 2009). Articles have shown that current vibration devices induce beneficial increases in bone mineral density (BMD), although diminished functional loading has been shown to decrease bone quantity in the case of bed rest patients or astronauts under microgravity conditions (Iwamoto et al., 2005; Semler et al., 2008).

The glucocorticoids (GC) are considered the most powerful anti-inflammatory and immunomodulating drugs, with a number of well-documented side effects (Cardinale and Rittweger, 2006; Lane, 2001), among which osteoporosis (OP) and fractures (Fx) are the most frequently described (Schake et al., 2002). The pathogenesis of GC-induced fractures are complex and have not yet been clarified, although its thought to be characterized by mechanisms involving bone metabolism at various levels (Cardinale and Rittweger, 2006; Lane, 2001; Schake et al., 2002; Bijlsma et al., 2005). GCs stimulate osteoclast-mediated bone resorption and reduce osteoblast-mediated bone formation, which results overall in increased bone resorption. GCs are responsible for increased osteoblast and reduced osteoclast apoptosis and therefore contribute to a reduction in bone mass (Canalis et al., 2004; Weinstein et al., 2004). Hormones, growth factors, cytokines, mechanical loading, nutrition, aging and other unidentified factors regulate osteoblastogenesis and bone formation. In particular, osteoblasts synthesize and secrete receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) to stimulate osteoclastogenesis (Boyle et al., 2003; Eghbali-Fatourechi et al., 2003; Cardile et al., 2012). RANKL is a member of the tumor necrosis factor (TNF) cytokine family, which is a ligand for osteoprotegerin (OPG) and functions as a key factor for osteoclast differentiation and activation, while OPG is a decoy receptor for RANKL.

The aim of this study was to investigate the expression in bone tissue and the plasma levels of RANKL and OPG after administration of a glucocorticoid (prednisolone) to induce osteoporosis pharmacologically in rats, and the outcomes of physical activity on the skeletal system by treadmill and vibration platform training. The animals were divided into 5 groups. For bone evaluations we used whole-body scans, histology and histomorphometric analysis. RANKL and OPG expression levels were evaluated by immunohistochemistry and biochemical analysis.

Materials and Methods

Animal experimental design

Forty 12-week-old male albino Wistar rats (Charles River Laboratories, Wilmington, MA, USA), with an average weight 240±20 g body weight were used and individually housed in stainless steel cages during the entire stabling period and were permitted free cage activity without joint immobilization. The animals were divided into 5 groups: 1: control rats (n=8); 2: rats with osteoporosis receiving prednisolone (n=8); 3: rats receiving prednisolone and treadmill training (n=8); 4: rats receiving prednisolone and vibration stimulation training (n=8); 5: rats receiving prednisolone, treadmill and vibration stimulation training (n=8). The drug, prednisolone 21-hemisuccinate sodium salt (Sigma-Aldrich), at a dose of 7 mg/kg was administered through a subcutaneous injection, once daily for 4 weeks, as previously described (Folwarczna et al., 2011). Group 3 rats after drug treatment were subjected to treadmill exercise for 12 weeks (Columbus Instruments, Columbus, OH, USA), five times a week for 30 minutes, with the treadmill inclined at 2° set at 10-speed m/min. The group 4 rats were subjected for 12 weeks to a vibrating plate following drug treatment. Group 5 rats were subjected to a combination of treadmill and vibrating plate for 12 weeks after treatment. Animals in the vibration group were placed in individual 13x28x15 cm compartments attached to a Pneu-Vibe vibration platform (Pneumex, Sandpoint, ID, USA). This group received 30 minutes of whole-body vibration each day, 5 days per week, for 12 weeks as previously described (Maddalozzo et al., 2008). The animals were maintained at 20-22°C, with a relative humidity of 40-60% and a photoperiod of 12=12 h, light and dark. The day following the last training, the animals were sacrificed as previously described (Musumeci et al., 2012c). All procedures conformed to the guidelines of the Animal Care and Use Committee of the University of Catania.

Whole-body scans

Body weight was recorded once a week. Bone mineral density (BMD) was measured in vivo for all animals throughout the whole body, spine and femurs by dual-energy x-ray absorptiometry (Lunar Prodigy, Lunar, Madison, WI, USA) at the beginning and after treatment. The rats were anesthetized with 2-3% isoflurane delivered in 100% oxygen (1.5 l min^{-1}) via a nose cone during the scanning procedure.

Histology

The femurs were explanted, cleaned from soft tissues, fixed in 10% buffered-formalin and decalcified

in 12.5 % ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) + 1.25% sodium hydroxide (NAOH) (Sigma-Aldrich) for approximately 3 weeks. The endpoint was determined by x-ray. Following overnight wash, specimens were treated as previously described (Musumeci et al., 2011d) and stained for routine histologic evaluation by Hematoxylin and Eosin (H&E) for the morphological structure and by Goldner's Trichrome for osteoid presence. The sections were observed with an Axioplan Zeiss light microscope (Germany).

Histomorphometric analysis

Histomorphometric analysis was performed on the total number of rats used and specifically on both medial and lateral femurs from untreated and treated animals, using a light microscope linked to image analysis, Kontron KS 300 software (Kontron Electronics, Eching bei Munchen, Germany). Bone volume and surface parameters were obtained by tracing relevant surfaces with a cursor on the video screen image using an automated mode. All sections were examined blindly. Bone volume of the diaphyseal, proximal metaphysis and epiphysis were measured on three nonconsecutive serial sections stained with von Kossa at a magnification of 100 μm. A standard area of three adjacent frames (0.78 mm^2 each) in the vertical axis, situated at least 1 mm from the growth plate to exclude the primary spongiosa, was used. Bone volume was defined as the percentage of metaphyseal bone area. Surface parameters were measured in two sections stained with toluidine blue at a magnification of 200 μm. A standard area of 18 frames (3 parallel to the growth plate and 6 in the vertical axis), measuring 0.18 mm^2 each, located in the secondary spongiosa in a corresponding area to that used for assessing bone volume, was used.

Fifteen fields randomly selected from each section were analyzed and the percent area stained with RANKL and OPG was calculated using an image analyzer (Image-Pro Plus 4.5.1, Immagini & Computer, Milan, Italy), which quantifies the level of positive immunolabelling in each field, as described previously (Rodella et al., 2010; Musumeci et al., 2011e). Digital pictures were taken using an Axioplan Zeiss light microscope and photographed using a Canon digital camera. Two blinded investigators, whose evaluations were assumed to be correct if values were not significantly different, made evaluations.

Immunohistochemistry

Sections were processed as previously described (Loreto et al., 2011b). Briefly, they were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were irradiated as previously described (Musumeci et al., 2012d). The sections were then incubated with diluted rabbit polyclonal RANKL antibody (Abcam Inc.Cambridge, MA USA), and goat polyclonal OPG (Santa Cruz Biotechnology, INC., California, USA), both diluted 1:100 in phosphate buffer saline (PBS), overnight at 4°C. The secondary antibody, biotinylated anti mouse antibody was applied for 30 minutes, at room temperature, followed by the avidin-biotin-peroxidase complex (Vector Elite Kit Abbott, Chicago, IL, USA) for the same period and conditions. The immunoreaction was visualized by incubating the sections for 4 minutes in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, CA, USA). The sections were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA) observed with an Axioplan Zeiss light microscope (Germany).

The RANKL-OPG-staining status was identified as either negative or positive. Positive staining was defined as the presence of a brown chromogen detection either on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm, or in the immediate lacunar/pericellular space. The proportion of immunopositive cells was assessed by light microscopy and expressed as the percentage of RANKL-OPG immunopositive cells (Extent Score=ES). This ES was independently evaluated by 3 investigators (2 anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: 0 = <5%; + = 5-30%; ++ = 31-50%; +++ = 51-75%, and ++++ = >75%. Counting was performed at x200 magnification. Positive and negative controls were performed as previously described (Loreto et al., 2012). Sections from rat bone marrow extract were used for both antibodies as positive control. The positive immunolabeling were both perinuclear and cytoplasmic. Sections of rat bone were randomly drawn from experimental samples for the negative control. These were then treated with normal rabbit serum instead of the specific antibodies.

Biochemical studies

RANKL (EIAab Science Co., Ltd., Wuhan, China) and OPG (Immunodiagnostic Systems Ltd., Bolton, UK) were measured using ELISA Assay kit according to the manufacturers' instructions for the quantitative determination of RANKL and OPG level in the plasma.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS® release 16.0, Chicago, IL, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's t test, whilst comparison between more than two groups was tested using analysis of variance

(ANOVA) and Bonferroni's test. P-values of less than 0.05 were considered statistically significant. Data are presented as the mean ± SEM. Cohen's kappa was applied to measure the agreement between the three observers and averaged to evaluate overall agreement using the following grading: 0-0.2 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.0 (almost perfect).

Results

Whole body composition

Control rats (group 1) showed a regular increase of their BMD that reached 166±7.4 mg/cm^2 in the whole body (+ 35% versus basal value); 151±8.4 mg/cm^2 at the spine (+ 42% versus basal value); and 140±11.4 mg/cm^2 at the femur (+48% versus basal value) (Table 1, Fig. 1). In prednisolone-osteoporosis rats (group 2), BMD was significantly decreased ($p<0.05$) in the whole body, spine, and femur and led to the development of osteoporosis within 4 weeks after drug administration (Table 1, Fig. 1). In groups 3 and 4, rats produced variation in BMD compared to control rats after 12 weeks of drug administration. BMD of rats receiving treatment and training regimes (group 5) showed no significant difference in their BMD, compared to control rats after 12 weeks drug administration (Table 1, Fig. 1).

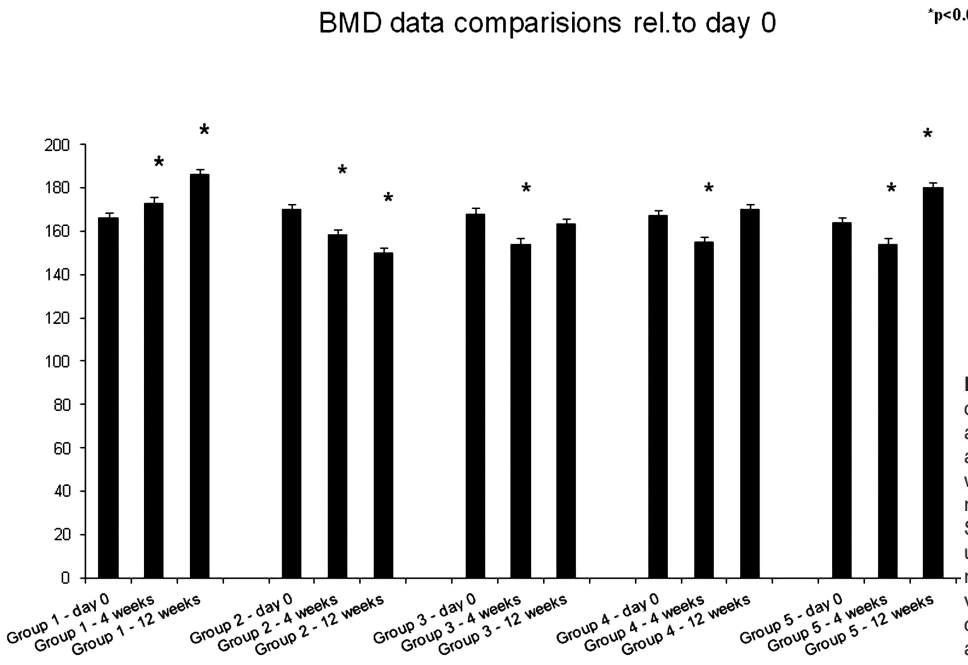

Fig. 1. Multiple comparisons of the effects of prednisolone in the total body, spine and femur at the start of drug administration after 4 weeks and after 12 weeks on bone mass and mineralization in rats. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. All data were normalized to day 0 values. * $p < 0.05$, when compared to the control group. Abbreviation: ANOVA, analysis of variance.

Table 1. Effects of prednisolone in the total body, spine and femur.

Parameter, group	Group 1	Group 2	Group 3	Group 4	Group 5
Start of drug administration, mg/cm^2					
Total body BMD	166±7.4	170±12.4	168±9.1	167±7.5	164±7.9
Spine BMD	151±8.4	152±6.6	155±8.4	155±6.1	152±9.3
Femur BMD	140±11.4	144±10.6	143±10.6	139±8.7	141±10.4
After 4 weeks of drug administration, mg/cm^2					
Total body BMD	173±6.3	158±7.8	154±3.5	155±4.0	154±6.9
Spine BMD	159±7.4	144±8.3	145±6.8	143±2.1	144±6.3
Femur BMD	148±8.6	137±6.9	137±9.5	141±7.7	139±3.7
After 12 weeks of drug administration and training, mg/cm^2					
Total body BMD	186±6.4	150±9.2	163±6.5	170±5.5	180±6.4
Spine BMD	170±7.3	137±4.3	149±8.8	159±6.6	167±7.3
Femur BMD	160±9.8	129±5.4	142±9.4	150±6.7	159±9.4

Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. *: $p<0.05$, when compared to the control group.

Histological observations

Control rats (group 1) showed a normal morphological structure with the absence of bone structural alterations, as observed by H&E staining (Fig. 2A,B). In Group 2 rats, the thickness of cortical bone was smaller compared to the control cortical bone (Fig. 2D,E). Goldner's Trichrome staining showed the differentiation of osteoid (red) from mineralized bone matrix (green) in control rats (Fig. 2C). Prednisolone-osteoporosis rats showed a decreased osteoid formation (Fig. 2F).

Histomorphometric analyses

Prednisolone caused a substantial decrease in bone volume (group 2). We found that the bone volume was increased by 12.9, 24.7, and 42.8% in animals belonging to experimental groups 3, 4 and 5, respectively, compared with group 2. This increase in bone volume in group 5 was significant compared with the bone volume of group 2 and comparable to the control group.

We did not find any significant differences in epiphysis, metaphysis and diaphysis areas of rat femoral bones. The immunohistochemical analysis showed that the antibodies (RANKL and OPG) were localized in different zones of bone (diaphysis, metaphysis and epiphysis) and in different bone cells (osteoblasts, osteocytes and osteoclasts, Fig. 3A-D).

Immunohistochemistry observations

RANKL was over expressed in bone cells after prednisolone treatment (group 2; Fig. 4B (ES = ++++)). In prednisolone and treadmill training treatment (group 3) the expression of RANKL showed strong immunostaining in bone cells (Fig. 4C. (ES = +++)), while weak immunostaining was observed in specimens treated with prednisolone and vibration stimulation training (group 4; Fig. 4D (ES = ++)). Weak immunostaining was observed in group 5 specimens (Fig. 4E) that were comparable to the control group (Fig. 4A (ES = +)). No immunoreaction was observed in the negative control treated with PBS without the primary antibodies (Fig. 4F). The percentage of RANKL-positive cells and the staining intensity were significantly greater ($P \leq 0.05$) in control bone compared with osteoporosis bone (Fig. 6A). Interobserver agreement, measured as Kappa coefficient, was 0.95 (almost perfect).

The expression of OPG was weak in bone cells after prednisolone treatment in group 2 (Fig. 5B (ES = +)) and in group 3 (Fig. 5C (ES = +)), while strong immuno-staining was observed in specimens treated with prednisolone and vibration stimulation training (group 4;

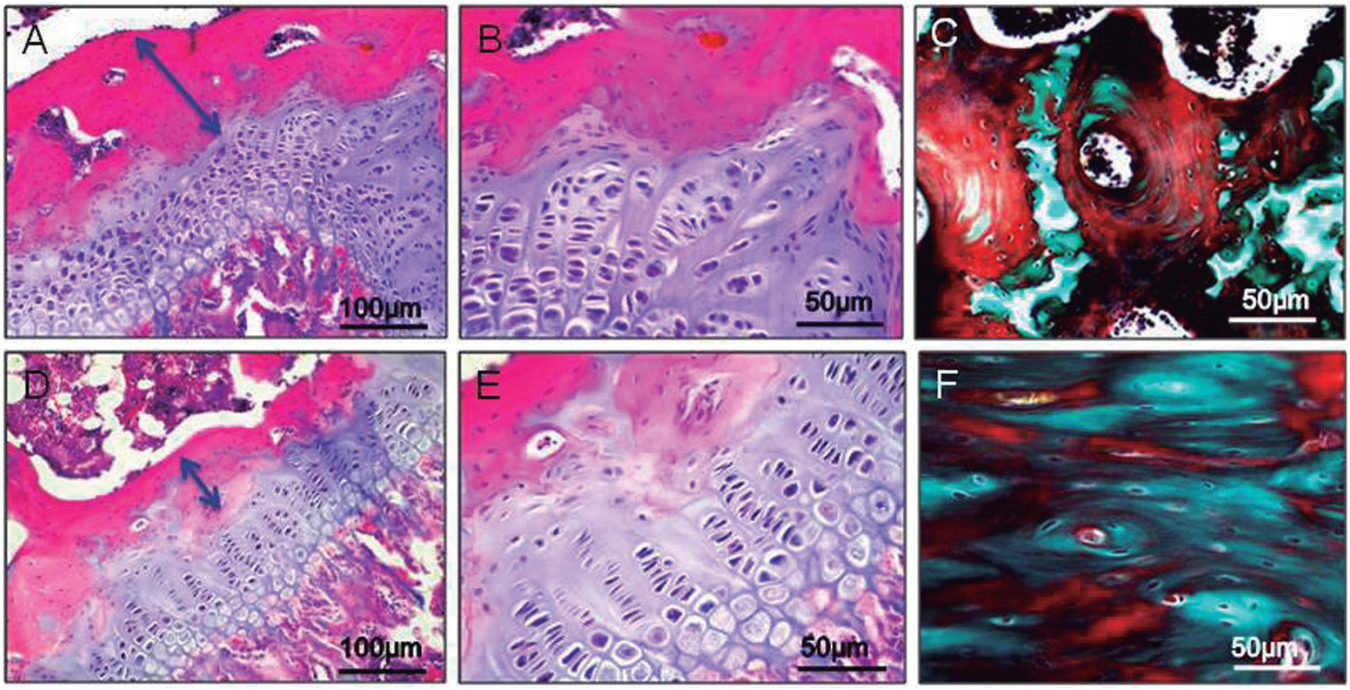

Fig. 2. A, B. H&E staining demonstrated a normal morphological structure with absence of structural alterations in control rats (group 1). **D, E.** H&E staining demonstrated in prednisolone-osteoporosis rats (group 2) the thickness of cortical bone is smaller compared to the control cortical bone (arrows). **C, F.** Goldner's trichrome staning was used to see the differentiation of osteoid (red) from mineralized bone (green) matrix in control rats **(C)** and in prednisolone-osteoporosis rats **(F)** where the osteoid formation decreased. Abbreviation: H&E, hematoxylin and eosin. Scale bars: A, D, 100 μm; B, C, E, F, 50 μm.

Fig. 5D (ES = ++)). Very strong immunostaining was observed in group 5 specimens (Fig. 5E) that were comparable to the control group (Fig. 5A (ES = +++)). No immunoreaction was observed in the negative control treated with PBS without the primary antibodies (Fig. 5F). The percentage of OPG-positive cells and the staining intensity were significantly greater ($P \leq 0.05$) in control bone compared with osteoporosis bone (Fig. 6B). Interobserver agreement, measured as Kappa coefficient, was 0.91 (almost perfect).

Biochemical studies

The plasma levels of RANKL significantly increased after prednisolone administration (group 2) in comparison to control (group 1). Rats receiving prednisolone and treadmill training (group 3) and rats receiving prednisolone and vibration stimulation training (group 4) increased the level of RANKL in comparison to control rats. The plasma levels of RANKL significantly decreased in rats receiving prednisolone, treadmill and vibration stimulation training (group 5) in comparison to the prednisolone-osteoporosis rats (group 2) equated to the controls (group 1); (Fig. 7A,C).

The plasma levels of OPG significantly decreased after prednisolone administration (group 2) in comparison to control (group 1). Rats receiving prednisolone and treadmill training (group 3) did not markedly affect plasma OPG levels. Rats receiving prednisolone and vibration stimulation training (group 4) significantly increased the level of OPG in comparison to control rats. The plasma levels of OPG significantly increased in rats receiving prednisolone, treadmill and vibration stimulation training (group 5) in comparison to

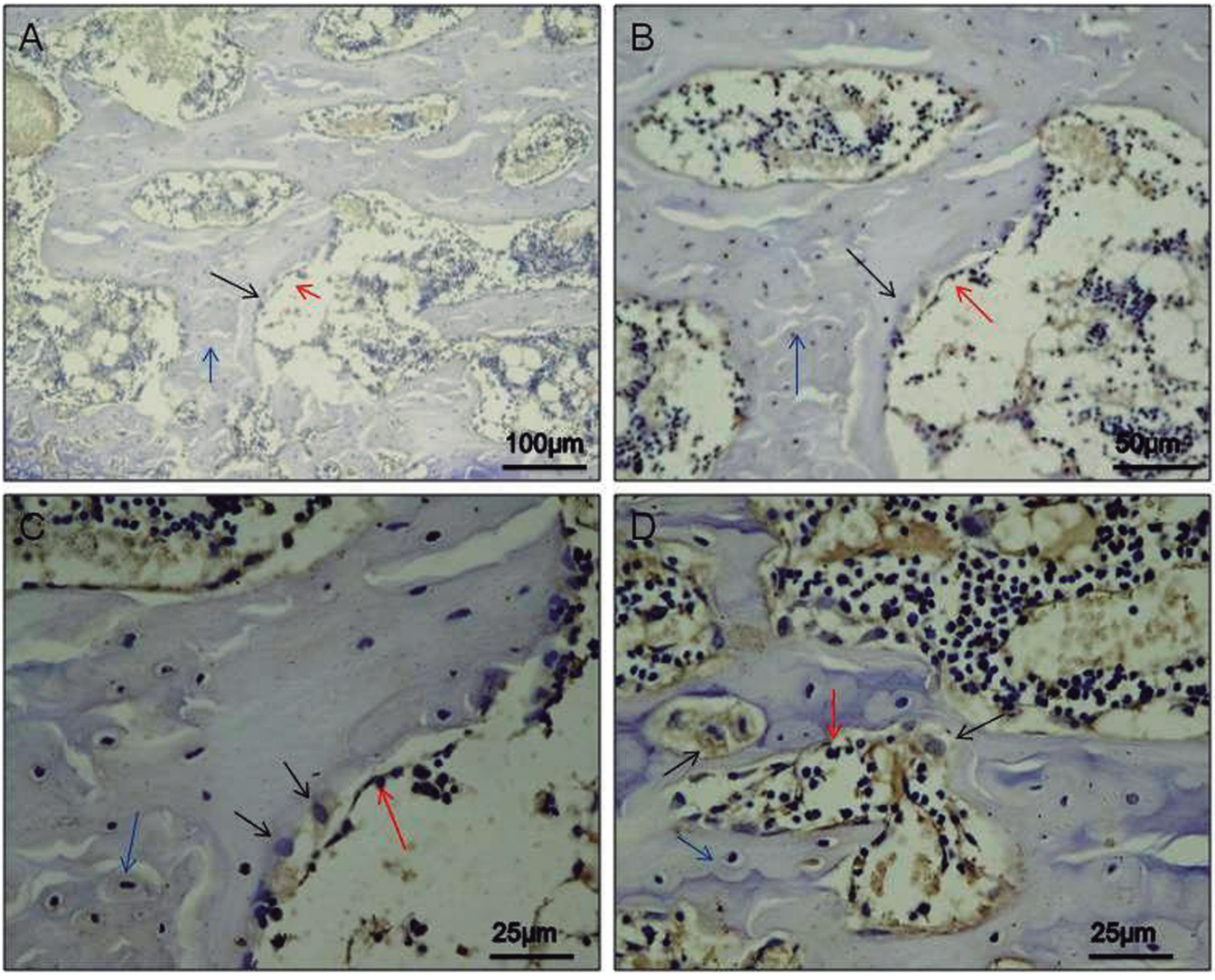

Fig. 3. A-D. Bone cells [Osteoblasts (red arrow), osteocytes (blue arrow), osteoclasts (black arrow)] from rat femoral bones. Scale bars: A, 100 μm; B, 50 μm; C, D, 25 μm.

the prednisolone-osteoporosis rats (group 2) equated to the controls (group 1); (Fig.7 B,C).

Discussion

The process of bone remodeling is characterized by a balance alternating between bone formation and resorption of the mineralized bone matrix. This process is made possible by the presence of two cell types: osteoblasts and osteoclasts. These cells, after cytokine and hormonal stimuli, express and produce a variety of protein molecules, which play an essential role in the regulation of osteoclastogenesis that lead to the process of bone remodeling. Both male and female are subjected to a gradual loss in bone density during the aging process, but females undergo a more rapid and marked bone loss after menopause, mainly due to declining estrogen levels. Recent studies suggest that the effects of estrogen deficiency on bone remodeling are in part due to activation of the RANKL/RANK/OPG system (Delmas, 2008). RANKL is a specific mediator belonging to the family of tumor necrosis factors (TNF) that are produced by osteoblasts due to various stimuli (e.g. hormones, cytokines and growth factors). RANKL is able to bind to a specific receptor (RANK) expressed on both progenitor and mature osteoclast cells. The interaction of RANKL with RANK results in the transformation of mononuclear precursors into mature osteoclasts (Boyle et al., 2003). This mechanism is essential for the formation, function and survival of osteoclasts and is a necessary condition for osteoclasts to perform their primary activity of bone resorption. OPG is a soluble factor produced by osteoblasts that is structurally similar to RANK. OPG acts as a receptor "decoy" binding RANKL before its interaction with RANK and actually blocks the biological activities. The activity of osteoclasts is dependent on the ratio of RANKL and OPG. When there is an excess of RANKL, resorption prevails, whilst excess OPG slows bone resorption down. In postmenopausal women, following the drop in estrogen levels, increased levels of RANKL are expressed by osteoblasts, suggesting that there is a change in the relationship between RANKL and OPG that favors bone resorption (Delmas, 2008) with negative consequences on homeostasis of the skeleton. Recent interesting studies demonstrated the impact of RANKL/OPG ratio in pathological osteolytic conditions in periodontal disease (Liu et al., 2003) and in osteolytic bone tumor (Huang et al., 2000). In the first study the ratio of RANKL to OPG in periodontitis has increased, this data suggested that up regulation of RANKL mRNA in both inflammatory cells and epithelium may be

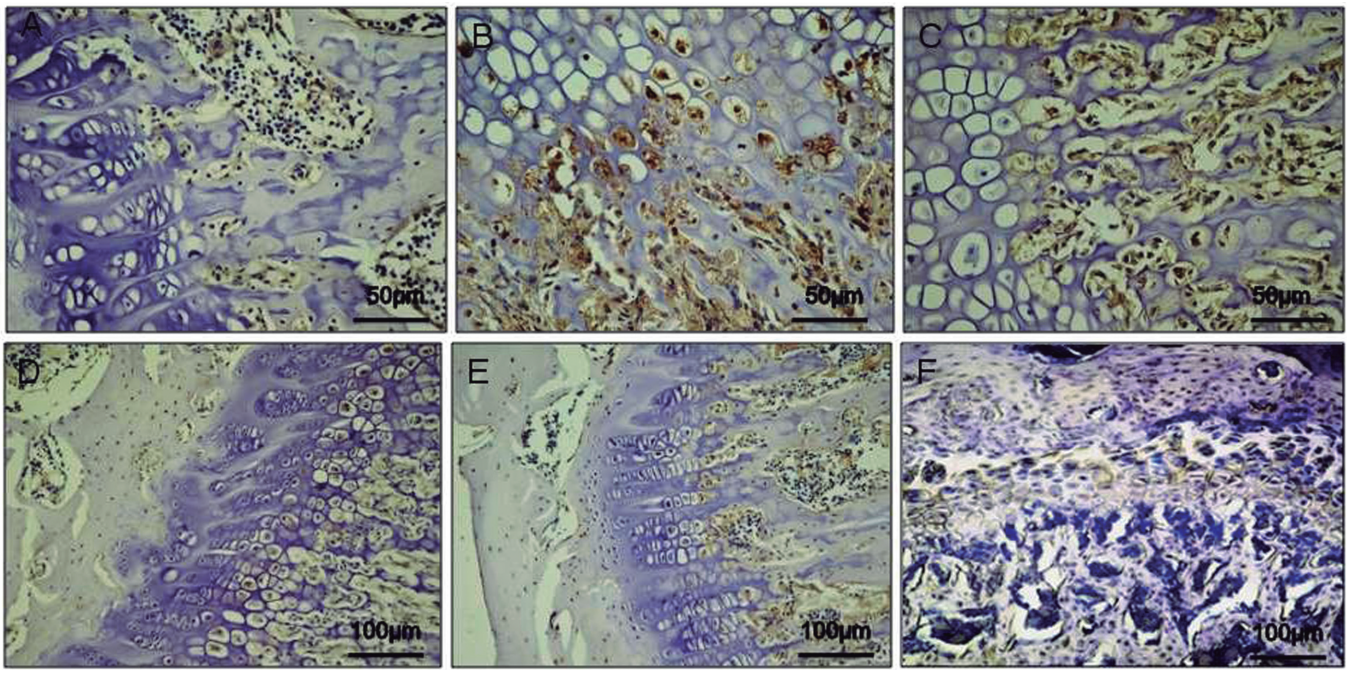

Fig. 5. A. RANKL immunohistochemistry specimen from control bone (group 1) exhibited a weak RANKL immunostaining in bone cells from rat femoral bones. Magnification x40; Scale bars: 50μm. **B.** RANKL was over expressed in bone cells from rat femoral bones after prednisolone treatment (group 2). **C.** In prednisolone and treadmill training treatment (group 3) the expression of RANKL showed a strong immunostaining in bone cells from rat femoral bones. **D.** Weak RANKL immunostaining was observed in specimens treated with prednisolone and vibration stimulation training (group 4) in bone cells from rat femoral bones. **E.** Weak RANKL immunostaining was observed in bone cells from rat femoral bones in specimens treated with prednisolone, treadmill training and vibration stimulation training (group 5). Specimens were comparable to the control group. **F.** No immunoreaction was observed in the negative control treated with PBS without the primary antibodies. Abbreviation: RANKL, receptor activator of nuclear factor (NF)-κB ligand. Scale bars: A-C, 50 μm; D-F, 100 μm.

associated with the activation of osteoclastic bone destruction in periodontitis. In the second study the results suggested that osteoprotegerin ligand (OPGL) also called RANKL, may be essential for the tumor cell to induce the formation of osteoclast-like giant cells in Giant cell tumor of bone (GCT). The ratio of OPGL/OPG produced by stromal-like tumor cells may contribute to the degree of osteoclast-like giant cell

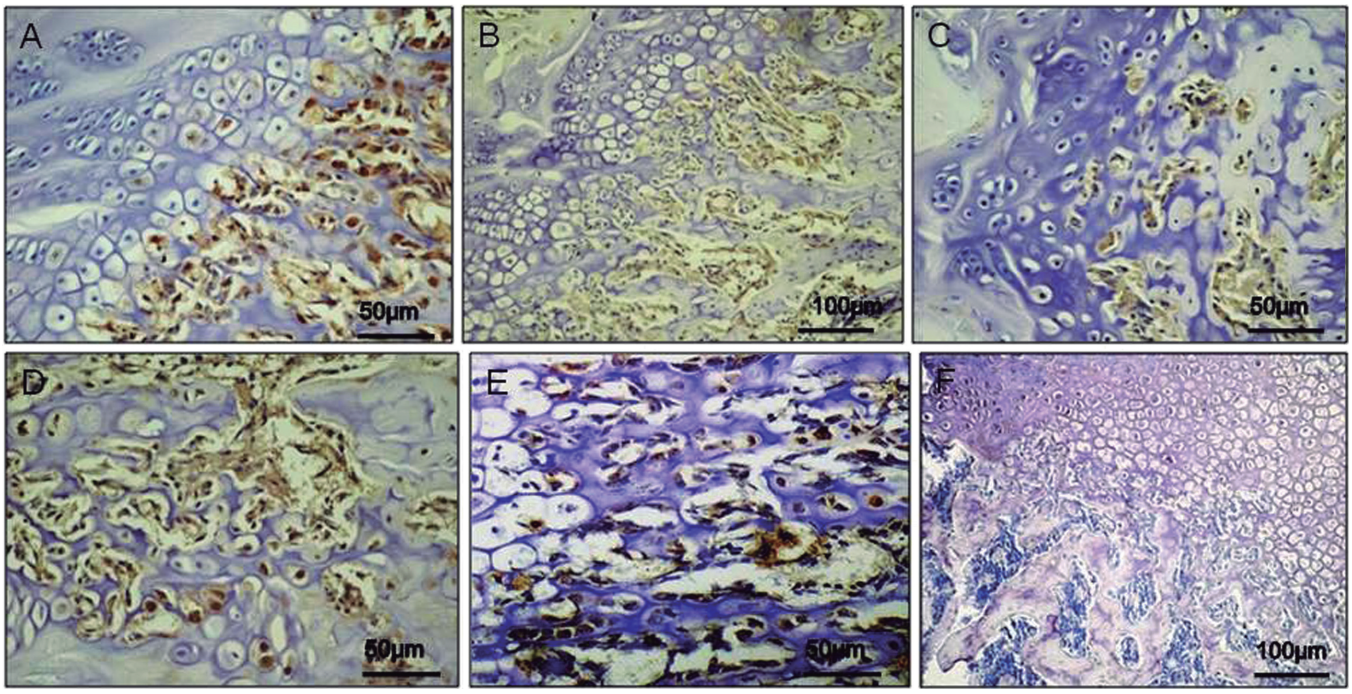

Fig. 6. A. OPG immunohistochemistry specimen from control bone (group 1) exhibited a very strong OPG immunostaining in bone cells from rat femoral bones. **B.** Weak OPG immunostaining was observed in bone cells from rat femoral bones after prednisolone treatment (group 2). **C.** Weak OPG immunostaining was observed in prednisolone and treadmill training treatment (group 3) in bone cells from rat femoral bones. **D.** Strong OPG immunostaining was observed in specimens treated with prednisolone and vibration stimulation training (group 4) in bone cells from rat femoral bones. **E.** Very strong OPG immunostaining was observed in bone cells from rat femoral bones in specimens treated with prednisolone, treadmill training and vibration stimulation training (group 5). Specimens were comparable to the control group. **F.** No immunoreaction was observed in the negative control treated with PBS without the primary antibodies. Abbreviations: OPG, osteoprotegerin. Scale bars: A, C-E, 50 μm; B, F, 100 μm.

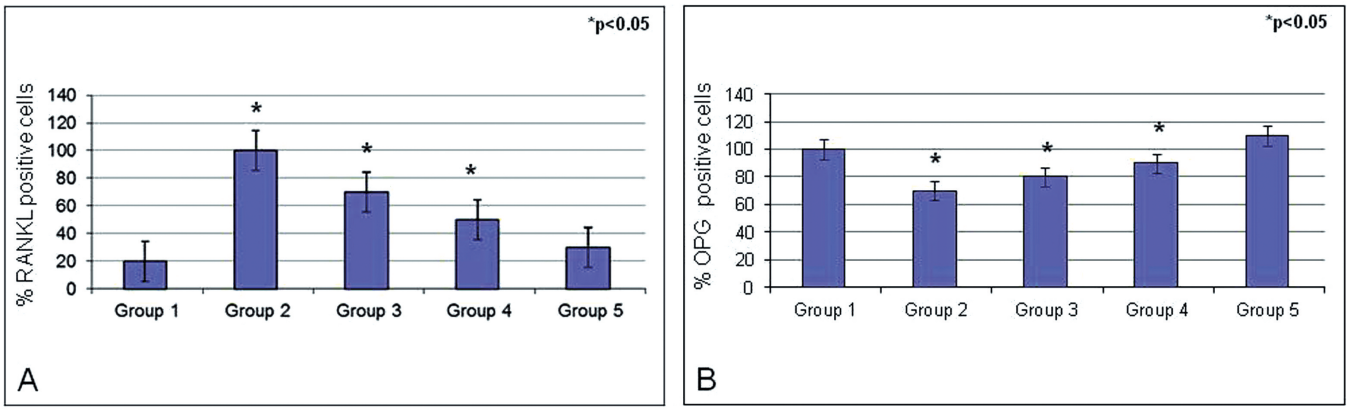

Fig. 7. A. Percentage of RANKL positive cells out of the total number of cells counted in control group and in osteoporosis groups. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. * p<0.05, when compared to the control group. Abbreviations: ANOVA, analysis of variance; RANKL, receptor activator of nuclear factor (NF)-κB ligand. **B.** Percentage of OPG positive cells out of the total number of cells counted in control group and in osteoporosis groups. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. * p<0.05, when compared to the control group. Abbreviations: ANOVA, analysis of variance; OPG, osteoprotegerin.

formation and bone destruction in GCT.

In our report the animals were divided into 5 groups, and after treatment was found a RANKL over expression in all bone cell types for rat femoral bones in groups 2 and 3. In the same groups (2 and 3) the expression of OPG was weak. In group 4, was found a weak RANKL immunostaining comparable to specimens receiving only prednisolone (group 2), whilst in group 5, the expression of RANKL was also weak comparable to the control group (group 1). Conversely, in group 4 was found a strong OPG immunostaining comparable to specimens receiving only prednisolone (group 2), whilst in group 5 the expression of OPG was also strong comparable to the control group (group 1). These data were also strengthened by biochemical analysis where RANKL levels significantly increased in groups 2 and 3 and decreased in groups 4 and 5. Conversely, OPG levels significantly decreased in groups 2 and 3 and increased in groups 4 and 5.

These results confirm the beneficial effect of physical activity (treadmill and vibration stimulation training) on the skeletal system and we can also confirm that suppression of bone formation is a central feature in the pathogenesis of glucocorticoid-induced osteoporosis. In the initial phase, at the onset of treatment, the reduction in bone mass (about 12%) is related to the stimulation of bone resorption through the activating cytokines, RANKL and OPG system, that plays a key role in osteoclast biology and bone remodeling (Manolagas and Weinstein, 1999; Hofbauer and Heufelder, 2001). GCs stimulate the expression by stromal cells/osteoblasts of RANKL, that binding its receptor RANK, expressed on the osteoclast, stimulates the differentiation and activation of osteoclasts and inhibits apoptosis. In addition, GCs inhibit the production OPG that is a soluble cytokine receptor, which acts as a "Trap" receptor due to its high affinity for RANKL, preventing the RANKL/RANK bond. On the contrary, in line with other authors that studied the effect of RANKL/OPG even if only in old and postmenopausal women (Marques et al., 2011; Bergström et al., 2012), we can assert that the effect of physical activity, in an in vivo rat model of osteoporosis, using treadmill and vibration stimulation training, improves the bone mass on the skeletal system (Fritton et al., 1997; Flieger et al., 1998; Rubin et al., 2003, 2004). Bone is a dynamic tissue capable of loading-induced structural adaptation through the process of bone remodeling. While this concept is well accepted, the underlying mechanism by which it is accomplished remains unclear. In vitro mechanical stimulation of osteocytes has been demonstrated to regulate the production of several soluble signaling molecules known to influence osteoblast and osteoclast activity, including

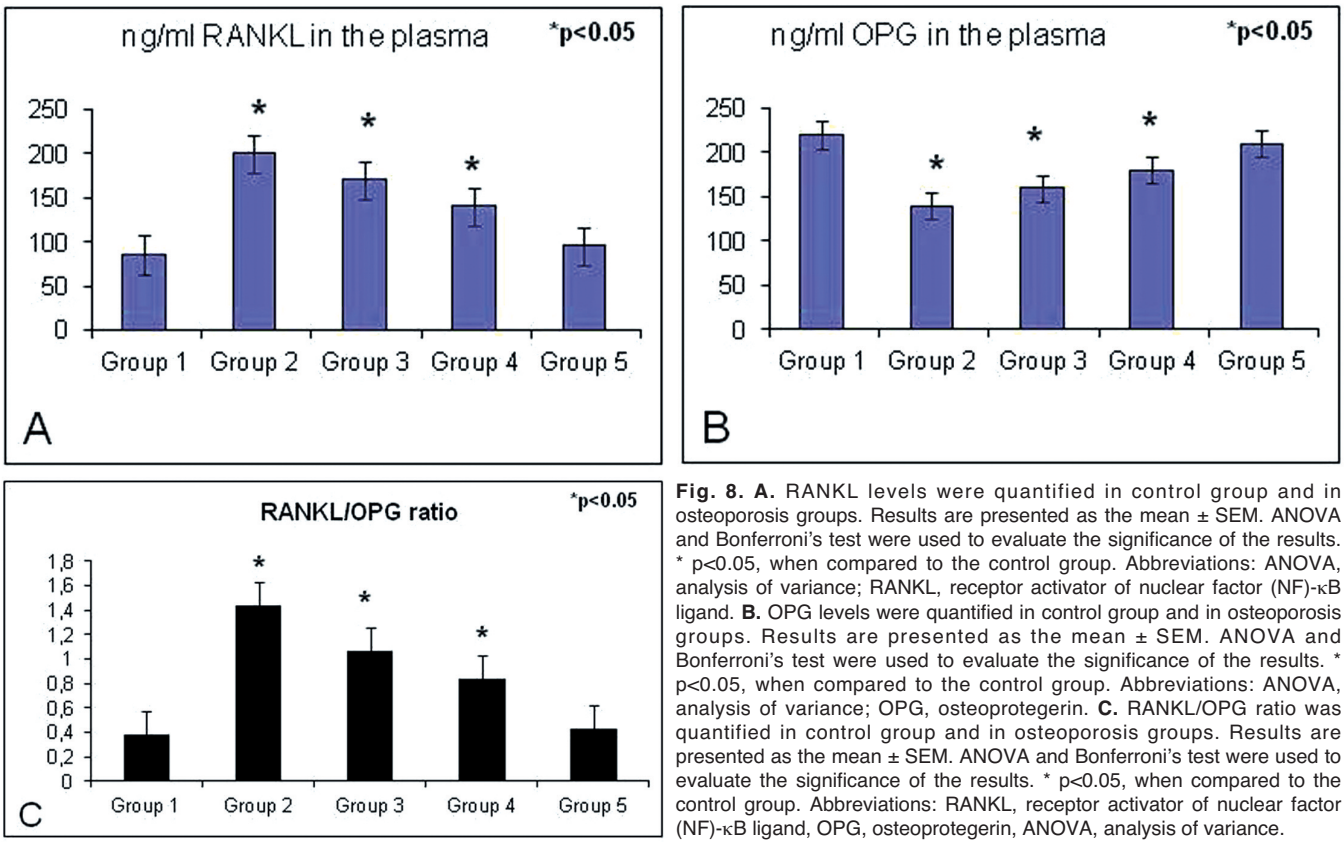

Fig. 8. A. RANKL levels were quantified in control group and in osteoporosis groups. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. * p<0.05, when compared to the control group. Abbreviations: ANOVA, analysis of variance; RANKL, receptor activator of nuclear factor (NF)-κB ligand. **B.** OPG levels were quantified in control group and in osteoporosis groups. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. * p<0.05, when compared to the control group. Abbreviations: ANOVA, analysis of variance; OPG, osteoprotegerin. **C.** RANKL/OPG ratio was quantified in control group and in osteoporosis groups. Results are presented as the mean ± SEM. ANOVA and Bonferroni's test were used to evaluate the significance of the results. * p<0.05, when compared to the control group. Abbreviations: RANKL, receptor activator of nuclear factor (NF)-κB ligand, OPG, osteoprotegerin, ANOVA, analysis of variance.

prostaglandin E2 (PGE2), RANKL, and OPG (Ajubi et al., 1999; Verschueren et al., 2004). They are the major signaling axis controlling osteoclast formation, activation and survival. Our results are in accordance with Lau et al., who measured RANKL and OPG mRNA expression changes in vibrated MLO-Y4 cells. Cells under vibration expressed a significantly lower level of RANKL (Lau et al., 2010). The mechanism behind the frequency dependent osteocyte response to vibration is not well understood. We can hypothesize that this effect also depends on the role of RANKL during the vibrating stimulations. A possible explanation is that, due to cellular viscoelasticity, the cells are less stiff and thus more deformable and responsive. However, at higher frequencies, the mechanical signal may excite bone cells via different mechanotransducing processes independent of cellular deformation (You et al., 2008).

In conclusion, our findings suggest that it might be possible, through mechanical stimulation, to inhibit the activity of RANKL. Mechanical stimulation could release inhibitors of RANKL such as OPG that are capable of inhibiting RANKL activity, preventing osteoblast differentiation into mature osteoclasts and thus inhibiting bone destruction. The balance between RANKL and OPG production establishes the level of osteoclastogenesis. So in certain diseases, such as osteoporosis, mechanical stimulation could be a possible therapeutic treatment, through lowering the RANKL level, thereby increasing bone formation and preventing fractures in osteoporotic bone. The present data are preliminary and so further and longer-term animal, in vitro and clinical studies are needed to understand the exact mechanism of production and regulation of RANKL in bone tissue under mechanical stimulations. This would provide new insights into the occurrence and progression of osteoporosis and may represent the biological basis for future attempts in medical therapy to preserve tissue function and prevent further bone damage.

Aknowledgements. This study was supported by grants provided by the Medicine and Surgery Faculty of Catania, Department of Bio-Medical Sciences, Section of Human Anatomy and Histology, University of Catania. The authors would like to thank Dr. Girish Pattappa from the AO Research Institute, Davos Platz, Switzerland, for commenting and making corrections to the paper.

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Accepted March 22, 2013