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Modulation of *in vitro* Osteoclast Activity Mediated by Different Doses of Parathyroid Hormone

Modulación *in Vitro* de la Actividad Osteoclástica Mediada por Diferentes Dosis de Hormona Paratiroidea

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SUMMARY: Bone remodeling is a process regulated by the interaction between cells and various molecules such as parathyroid hormone (PTH). The aim of the study was to evaluate the effect of different doses of PTH on osteoclast activity in a culture model of bone organs. Six-day-old male C57BL/6 mice (n=14) were euthanized and the calvariae were dissected and sectioned in the middle, keeping the periosteal and endosteal. The bone fragments were divided into three groups: Group I (control - without adding PTH), Group II (addition of 3 nM PTH) and Group III (30 nM PTH), all cultured in aMEM for up to 72 h osteoclast activity was evaluated by biochemical quantification of calcium released in the culture medium at intervals of 24, 48, and 72 h and by histomorphometric analysis of bone resorption lacunae at 72 h our results show that group II exhibited significantly higher values of calcium levels in the medium compared to group I ($p<0.05$) in all intervals, also being higher for group III at 24 hours ($p<0.05$). Group II promoted a greater demineralization area (22068 ± 2193 mm^2) than those found in group I (2084 ± 38 mm^2) and group III (8952 ± 246 mm^2), with statistically significant difference ($p<0.001$) among all groups. We concluded that in culture model of bone organs PTH promotes higher bone resorption when administered in lower doses.

KEY WORDS: Parathyroid hormone; Osteoclast; Bone remodeling; Organ culture techniques.

INTRODUCTION

Bone is a mineralized tissue that gives multiple mechanical and metabolic functions to the skeleton. This tissue is formed through the proliferation and differentiation of mesenchymal stem cells (MSCs) along a multi-step osteogenic pathway, which is controlled by a series of events involving gene regulation by many hormones, cytokines and growth factors and includes the crosstalk between multiple cell types for the formation and bone remodeling (Peng *et al*., 2016).

Parathyroid hormone (PTH) acts on bone and kidneys by controlling serum levels of calcium and phosphorus. It is a hormone composed of 84 amino acids, whose biologically active fraction corresponds to the 1-34 amino acid sequence. PTH related peptide (PTHrP) is a polypeptide that shows significant homology to PTH. Both PTH and PTHrP initiate their effects by binding with equal affinity to the same G-protein-coupled PTH receptor 1 (PTH1R), which is expressed on the surface of osteoblasts and osteocytes in bone, and tubular cells in the kidney (Silva & Bilezikian, 2015).

In bone tissue, PTH increases osteoclast formation and bone resorption by regulating the receptor activator of nuclear factor kappa B ligant (RANKL)/osteoprotegerin (OPG) expressed in osteoblasts. Moreover, it is also reported that PTH may have an anabolic effect on bone tissue, which increases bone mass (Del Fattore *et al*., 2008).

Elucidation of pathophysiology of this hormone excess (causing severe bone loss) and disability (promoting hypocalcemia) were unanimities for many decades (Potts, 2005). With the progress of purified PTH peptides tests in humans, a paradoxical effect of hormones was observed in the bone: administration of PTH increases bone mass, especially in trabecular bone, increases bone strength and reduces the incidence of fractures (Dempster *et al*., 2001; Neer *et al*., 2001). However, the protocol of administration is crucial for the anabolic action of PTH, since continuous infusion of the same dose of the hormone or large doses of one of its biologically active fragments promotes the predominance of

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resorptive action over the anabolic action (Horwitz *et al*., 2000).

Some critical steps in PTH action that regulates specific cellular responses which cause an anabolic effect on the skeleton still need to be established. This article aims to evaluate the effect of two different doses of PTH on *in vitro* osteoclast activity using a culture model of mice bone organs.

MATERIAL AND METHOD

Bone organs culture. The project was approved by the Ethics Committee on the Use of Animals of the Federal University of Rio Grande do Norte (CEUA-UFRN/Protocol number 008/2013). Six-day old male C57BL/6 mice (n=14) were euthanized via cervical dislocation and the calvariae were obtained with the aid of a stereoscope and dissected in sterile conditions under a laminar flow, covering the frontal and temporal bones and preserving the periosteum and the endosteal. The specimens were sectioned in half, totaling 28 bone fragments that were individually cultured in 24-well plates containing 300 uL of a-MEM without nucleosides (Cultilab, Brazil) and supplemented with 0.05 mg/mL gentamycin (Gibco, USA), 0.25 mg/ml amphotericin B (Gibco) and 15 % heat-inactivated horse serum (Sigma, USA). Group I (control) followed the normal culture conditions and 3 nM and 30 nM PTH (CIHR Matrix Dynamics, University of Toronto, Canada) were added to Groups II and III, respectively. Al the samples were kept at 37 °C with 5 % CO_2 and 95 % humidity for up to three days.

Biochemical analysis. Biochemical analyses were performed in triplicate at intervals of 24, 48 and 72 h Osteoclast activity was evaluated by dosing the calcium level in the medium culture collected at each interval using Total Calcium LiquiColor kit (Stanbio, USA) and reading absorbance in a spectrophotometer at 550 nm.

Histomorphometric evaluation. In the last interval (72 hours), the specimens (n=3/group) were fixed in 4 % paraformaldehyde and stained with Alizarin Red for microscopic evaluation of the bone resorption areas under confocal microscopy (Leica Microsystems, Germany). Images of five random fields were obtained from each specimen and the areas of osteoclastic resorption were assessed with the aid of ImageJ software (NIH, USA).

Statistical analysis. Data on biochemical levels of calcium and measures of bone resorption areas were subjected to normal testing and then to non-parametric statisticaltests of Kruskal-Wallis and Mann Whitney, considering a significance level of 5 % ($p<0.05$).

RESULTS

Figure 1 shows the measurements of the calcium concentration in the medium of the three groups over the experiment. In the groups cultured with PTH, the calcium concentration increased when the PTH exposure time was longer. The addition of a lower dose of PTH (Group II) promoted a significant increase ($P<0.05$) in calcium levels in all intervals, when compared to control (Group I). Statistically significant differences were found between control (Group I) and higher dose of PTH (Group III) only at 48 and 72 hours ($p<0.05$). When comparing the experimental

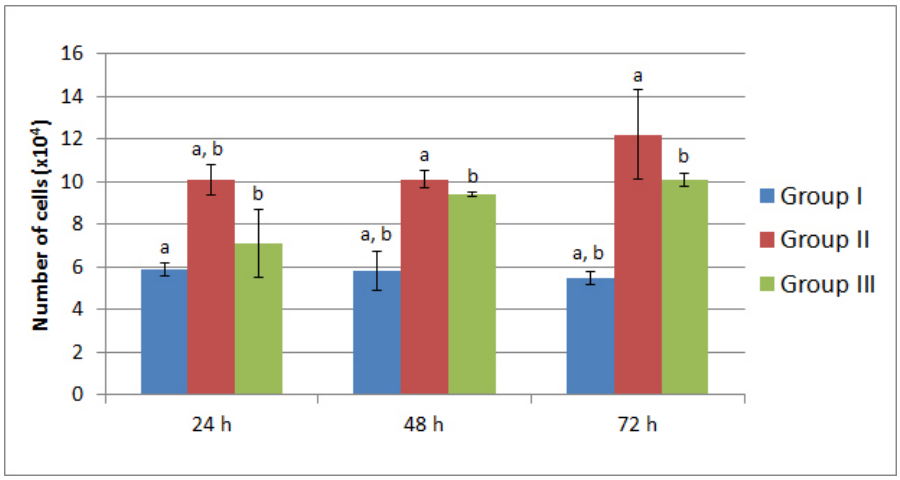

Fig. 1. Means ± standard deviation of calcium concentration in the medium at the intervals 24, 48 and 72 hours. Equal letters indicate statistically significant differences ($p<0.05$) between two groups in the specific interval. Mann Whitney test.

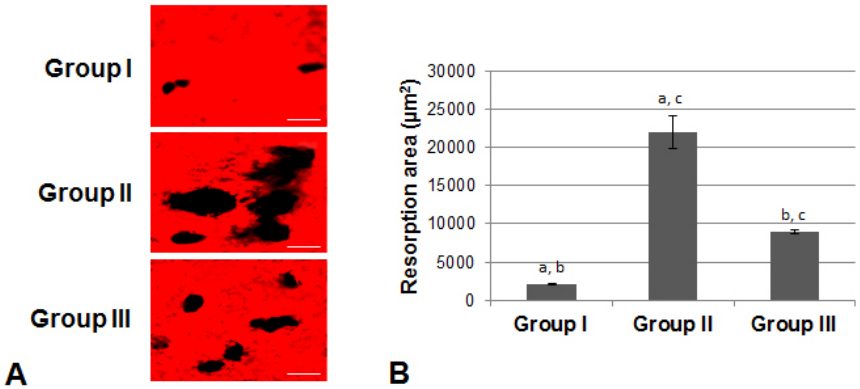

Fig. 2. (A) Photomicrograph of areas of bone resorption in calvariae cultured in the absence (Group I) or presence of PTH (Groups II and III) for 72 hours. (Alizarin Red staining; bar = 50 µm); (B) Means ± standard deviation of the areas of resorption by histological field evaluated at 72 h equal letters indicate statistically significant differences: (a) $p<0.001$; (B) $p<0.001$; (C) $p<0.001$. Mann Whitney test.

groups receiving different doses of PTH (Groups II and III), a statistically significant difference ($p<0.05$) was found only at an interval of 24 h.

In microscopy (Fig. 2A), the resorption lacunae promoted by a lower dose of PTH (Group II) were more numerous and larger than those promoted by higher dose of PTH (Group III). Figure 2B shows average values of the resorption areas for each histological field (n=5) of each specimen at a 72 h interval. Data show that Group II exhibited larger areas of resorption (22068 ± 2193 mm^2) than those observed in Group III (8952 ± 246 mm^2) and Group I (2084 ± 38 mm^2), with statistically significant differences ($p<0.001$) between all groups.

DISCUSSION

The anabolic effects of parathyroid extracts on bone were described in the 1930s (Selye, 1932). By the 1970s, the in vivo anabolic effects of PTH were observed when the hormone was injected in animals (Kalu *et al*., 1970), leading to the theory that the anabolic action of PTH would be greater than its resorptive action. PTH would increase bone mass by increasing the recruitment and activation of BMU (basic multicellular units), which operates in conjunction with the osteoblast precursors to promote their differentiation, inhibiting apoptosis of osteoblasts and osteocytes (Jilka, 2007) and the production of the bone formation inhibitor sclerostin (Keller & Kneissel, 2005). However, brief exposures and higher concentrations than the average PTH would be necessary to exert its "anabolic" effect (Poole & Reeve, 2005).

Fetal and neonatal bone organ cultures are often used for biochemical analysis of bone remodeling, in which the local interaction between osteoblastic and osteoclastic cells is maintained within the context of a three-dimensional bone matrix (Suzuki *et al*., 2005). However, few studies have examined the cellular response to factors that modulate the activity of bone cells. Experiments carried out *in vitro* in tissue culture (rat calvariae) showed that the addition of PTH to the culture medium inhibited collagen synthesis and increased bone resorption, bringing confusion to the issue (Dietrich *et al*., 1976).

The present study demonstrated that the addition of different doses of PTH in a model of mice bone culture produced larger (Group II) and smaller (Group III) areas of bone reabsorption. In fact, the different actions of PTH on bone formation and resorption have been intensively studied and new aspects of its connection with the receptor and the intracellular signaling pathways in osteoblasts have been described. PTH receptors have been identified in osteocytes, osteoblasts and its precursors - pluripotent mesenchymal progenitor cell line (Swarthout *et al*., 2002). On the other hand, PTH receptors were not identified in osteoclasts or their precursors, indicating that all the effects of PTH on bone resorption are mediated by osteoblasts (Strewler, 2001).

Furthermore, accumulated evidence shows that some of the PTH actions on bone tissue are mediated by direct effects of the hormone in osteocytes, the most abundant cells in bone (Bellido *et al*., 2013). PTH downregulates the expression of Sost, which encodes sclerostin, a potent inhibitor of bone formation mediated by T-cell–produced Wnt10b that is expressed by osteocytes (Bellido *et al*., 2005; Keller & Kneissel; Li *et al*., 2014). PTH also increases the expression

of growth factor fibroblast 23 (FGF23), protein expressed osteocytes (and osteoblasts) that regulate phosphate reabsorption in the kidney, contributing to mineral homeostasis (Rhee *et al*., 2011; Bellido *et al*., 2013).

Osteoblasts and stromal cells are involved in osteoclastogenesis through their cell-cell interactions with osteoclast progenitors (Ma *et al*., 2001). One of the key factors for the development of functional osteoclasts is the induction of cytokines, especially the receptor activator of nuclear factor kappa B ligant (RANKL) and its soluble decoy receptor osteoprotegerin (OPG) (Nakashima & de Crombrugghe, 2003). RANKL binds to RANK both on the surface of hematopoietic precursors of osteoclasts, promoting their differentiation and survival, and in fully formed osteoclasts, stimulating their activity. OPG inhibits RANKL induced bone resorption by binding to RANKL, and thereby preventing its access to the receptor RANK (Silva & Bilezikan).

Our results show that lower doses of PTH promote higher bone resorption. One of the possibilities for explaining this feature is related to RANK/RANKL/OPG pathway. In vivo studies have demonstrated that continuous infusion of PTH triggers a decrease of OPG and an increase of RANKL capable of inducing osteoclast differentiation and promoting bone resorption (Ma *et al*.). Another reason for our results would be the increased expression of a3 and d2 subunits of vacuolar-type H + adenosine triphosphatase (V-ATPase), which accelerates intracellular acidification when activated and increases bone resorption capability of osteoclasts (Liu *et al*., 2016).

The results of this study corroborate the findings of Suzuki *et al*., showing that the *in vitro* addition of low doses of PTH to the culture medium promotes an increase in the resorptive activity of osteoclasts, and suggests that the activities of osteoblasts and osteoclasts are coupled.

Jacome-Galarza *et al*. (2011) administered daily doses of PTH in rats for 7 and 14 days, and observed an increase in osteoclastogenesis *in vitro* under stimulation of RANKL and macrophage colony-stimulating factor (M-CSF), which was also observed in Group II of this study. The authors suggest that pre-treatment PTH in vivo indirectly affects osteoclast precursors, making them more responsive to RANKL and M-CSF, which increases activation of osteoclasts and thus justifies bone resorption observed *in vitro* assays.

In this study, it was observed that Group III decreased the osteoclastogenic effects showed by calcium concentration and the means of the areas of resorption when compared with Group II. These results corroborate the findings of Jilka *et al*. (2010), where the continuous administration of PTH or RANKL in mice showed that the cancellous bone mass was maintained in groups that received PTH, but was reduced in mice receiving RANKL, indicating that maintaining balanced remodeling requires osteoblastogenic effects in addition to those mediated by osteoclast.

A study in mice with conditional deletion of the PTHrP in osteocytes showed that PTH failed to increase RANKL expression and thereby osteoclastogenesis, demonstrating that PTHrP is necessary for full anabolic and catabolic bone responses to PTH administration. The PTHrP probably participated in the pathways of both experimental groups in this study, with Group II having greater resorption lacunae and Group III showing decreased osteoclastogenic effects (Saini *et al*., 2013).

PTH pro-osteoclastogenic actions are associated with upregulation of RANKL and OPG downregulation. However, the particular stage of differentiation of the target cell that supports osteoclastogenesis mediated by PTH remains undefined (Ben-awadh *et al*., 2014). Taken together, the data from this study suggest that high doses of PTH can activate different pathways from those of PTH in low doses, possibly via RANKL blocking by OPG, which would explain the lower bone resorption found in the specimens treated with higher PTH doses. Further research is required to elucidate the anabolic action mechanisms of PTH, aiming future clinical and therapeutic applications in the regulation of calcium homeostasis and bone resorption.

Taken together, the results of this work showed that in culture of bone organs PTH promotes greater bone resorption when administered at lower doses. The use of bone organ cultures associated to different molecules and bone metabolism markers may be considered an advance in establishing new directions in developing future preclinical studies on the therapy of bone disorders.

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GINANI, F.; MOTA-FILHO, H. G.; VASCONCELOS BARRETO, M. P. & BARBOZA, C. A. G. Modulación *in vitro* de la actividad osteoclástica mediada por diferentes dosis de hormona paratiroidea. *Int. J. Morphol., 35(2)*:584-588, 2017.

RESUMEN: La remodelación ósea es un proceso regulado por la interacción entre las células y varias moléculas como la hormona paratiroidea (PTH). El objetivo de este estudio fue evaluar el efecto de diferentes dosis de PTH sobre la actividad de los osteoclastos en un

modelo de cultivo de órganos óseos. Se sacrificaron ratones C57BL/6 machos, de 6 días de edad (n = 14), y se disecaron y seccionaron las calvarias, manteniendo el periostio y endostio. Los fragmentos óseos se dividieron en tres grupos: Grupo I (control - sin adición de PTH), Grupo II (adición de 3 mM de PTH) y Grupo III (30 nM de PTH), todos cultivados en aMEM hasta 72 horas. La actividad de los osteoclastos se evaluó mediante la cuantificación bioquímica de calcio liberado en medio de cultivo, a intervalos de 24, 48 y 72 horas, y por análisis histomorfométrico de las lagunas de resorción ósea a las 72 horas. Nuestros resultados muestran que el grupo II exhibió valores significativamente más altos de calcio en el medio, comparado con el grupo I ($p < 0.05$) en todos los intervalos, siendo también más alto para el grupo III a las 24 horas ($p < 0.05$). El grupo II promovió una mayor área de desmineralización (22068 ± 2193 mm^2) que los encontrados en el grupo I (2084 ± 38 mm^2) y en el grupo III (8952 ± 246 mm^2), con diferencia estadísticamente significativa ($p < 0,001$) entre todos los grupos. Concluimos que en el modelo de cultivo de órganos óseos la PTH promueve una mayor resorción ósea cuando se administra en dosis más bajas.

PALABRAS CLAVE: Hormona paratiroidea; Osteoclastos; Remodelación ósea; Técnicas de cultivo de órganos.

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