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Original Article

Host defense peptides clavanins A and MO reduce *in vitro* osteoclastogenesis

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Aim: Several systemic diseases, such as periodontitis and apical periodontitis, can cause extensive bone resorption. Host defense peptides may have the potential for the development of novel therapies for the bone resorption process. This study evaluated the potential of host defense peptides clavanins A, MO, and LL-37 in *in vitro* osteoclastogenesis. **Methods:** RAW 264.7 cultures were stimulated with recombinant of receptor activator of nuclear factor kappa B ligand in the presence of different tested concentrations of host defense peptides, besides calcium hydroxide and doxycycline. Cellular viability, nitric oxide production, and a number of differentiated osteoclast-like cells were also evaluated. **Results:** Results showed that none of the substances were cytotoxic, except for 128 $\mu g.mL^{-1}$ of doxycycline after 3 days. Host defense peptides, calcium hydroxide, and doxycycline did not interfere in nitric oxide production or downregulated it. An exception was observed in the presence of 2 $\mu g.mL^{-1}$ of doxycycline, in which nitric oxide production was up-regulated. All host defense peptides were capable of reducing osteoclast-like cell differentiation. **Conclusion:** Host defense peptides clavanins A and MO demonstrated to be potential suppressors of osteoclastogenesis *in vitro* without interfering in cellular viability and nitric oxide production. These promising results need to be further analyzed in *in vivo* models of bone resorption.

Keywords: Bone resorption. Antimicrobial cationic peptides. Nitric oxide. Osteogenesis.

Introduction

Bone remodeling is a process balanced between osteoblast-mediated bone deposition and osteoclast-developed bone resorption. Many oral diseases are mediated by an inflammatory process, increasing the recruitment of osteoclasts and enhancing bone erosion[1]. Periodontal disease and apical periodontitis present bone resorption with high osteoclast formation or hyperactivation, overcoming bone formation, and decreasing osteoblast activity[2].

Inflammatory conditions, such as local osteolysis, can be associated with inducible nitric oxide synthase (iNOS) activation[3]. NO can also promote cytokine production and bone turnover besides indirect induction of bone resorption[3]. In this regard, periodontitis results in higher production of NO compared to healthy gingiva[4]. Periodontal treatment may involve the use of several systemic antibiotics such as tetracycline (minocycline and doxycycline) as adjuvants due to its local distribution. It was demonstrated that doxycycline hyclate gel (local therapy) could aid in scaling and root planning in patients with moderate to severe chronic periodontitis, but the benefit is still uncertain[5]. For the endodontic treatment, the use of calcium hydroxide ($Ca(OH)_2$) as a local antimicrobial is accepted worldwide as intracanal dressing[6].

Despite the high success rate in existing periodontal and endodontic therapies, there are some limitations, mainly in tissue repair activity[6]. To improve bone repair in these diseases, it is essential to develop new substances. New therapies involving a direct effect on the bone can prolong the maintenance of the tooth in the oral cavity due to tissue support health. Host defense peptides (HDPs) are biomolecules from many organisms released in early defense response to infection and invasion by bacteria and other microorganisms[7]. HDPs may possess antimicrobial and immunomodulatory properties besides tissue repair induction[8]. In this context, human cells can be potential sources of HDPs[7]. Clavanin A is a promising HDP due to its known antibacterial, immunomodulatory, antitumor, and antiviral activities[9]. Besides, clavanin A was used as a model to create clavanin MO. Five hydrophobic amino acid residues (FLPII) were added to the N-terminus, being selected based on a computational search of the conserved region of other peptides with higher immunomodulatory activities[10]. It has been shown that different HDPs could improve therapies in the dental field.

Some peptides have been reported as having the potential to inhibit osteoclastogenesis such as LL-37[11], human beta-defensin-3 with C-terminal end contains a 15-amino acid polypeptide (HBD3 - C15)[12], synoeca-MP,[13] and HHC-10[13]. A previous study demonstrated that LL-37 and clavanins A and MO can modulate the inflammatory response of active cytokines presented in the osteoclastogenesis process, such as TNF-α, while $Ca(OH)_2$ up-regulated the IL-6 and IL-1α production[14]. This fact leads us to believe that clavanins A and MO may have the potential to inhibit osteoclastogenesis, a fact that has not yet been evaluated. Thus, this study aims to evaluate the biotechnological potential of HDPs clavanin A and MO in the oral osteoimmunological context and their capability to reduce *in vitro* osteoclastogenesis,

compared to LL-37 (HDP control), $Ca(OH)_2$ (used in the endodontic treatment) and doxycycline (used in the periodontal treatment).

Material and Methods

Peptide synthesis

Clavanin A (VFQFLGKIIHHVGNFVHGFSHVF-NH2), clavanin MO (FLPIIVFQFLGKI-IHHVGNFVHGFSHVF-NH2), and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFL-RNLVPRTES-NH2) were synthesized and purified (>95% purity) by Peptide 2.0 Inc. (USA). Molecular mass and purity of all peptides were analyzed by Matrix-Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry on an Auto-Flex III Speed instrument (Bruker Daltonics, Billerica, MA). Peptides were diluted in ultrapure water and quantified by UV absorption at 205, 215, and 225 nm, according to Murphy and Kies[15].

Doxycycline and calcium hydroxide preparation

$Ca(OH)_2$ (Iodontosul, Porto Alegre, Brazil) was weighed and diluted in ultrapure water before each experiment. Doxycycline (Pharmac, Brasilia, Brazil) was handled in capsules (100 mg in each unit). The capsules were opened, and doxycycline was weighed and diluted in ultrapure water before each experiment.

Cell culture, experimental groups and osteoclasts

Osteoclast precursor RAW 264.7 cell line (RAW; BCRJ code 0212; RRID CVCL_0493 – Rio de Janeiro, Brazil) is composed of monocytes derived from tumors induced in male BALB/c mice (*Mus musculus*), infected with murine leukemia Abelson virus[16]. RAW cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, California, USA) supplemented with 10% fetal bovine serum (Gibco, California, USA), 1% penicillin/streptomycin (1000 $U.mL^{-1}$) (Gibco, California, USA), 1% nonessential amino acid solution (Gibco, California, USA), 1% L-glutamine (Gibco, California, USA) and 0.1% gentamicin (Gibco, California, USA). Cell cultures were maintained in an incubator containing 5% CO_2 at 37°C and 95% humidity. Experiments were conducted with $2.5x10^3$ cells per wells in 96-well plates (Kasvi, China), stimulated with or without rRANKL 100 $ng.mL^{-1}$ (Peprotech, New Jersey, USA) and HDPs clavanin A, clavanin MO, and LL-37 (2, 8, 32, and 128 $\mu g.mL^{-1}$). Peptide stimulated cultures were compared to doxycycline and $Ca(OH)_2$ (2, 8, 32, and 128 $\mu g.mL^{-1}$). The concentrations were based on a previously published result[14]. Cell viability assay and NO production were analyzed after 3 and 7 days of cell culture. Half of the culture medium and stimuli were changed every 3 days. After 7 days, TRAP staining was performed, and the number of differentiated osteoclast-like cells was determined.

Cytotoxicity analyses

Peptides, $Ca(OH)_2$, and doxycycline cytotoxicity were analyzed by MTT colorimetric assay (Sigma-Aldrich, St. Louis, USA), read in a microplate reader (Bio-Tek Power Wave HT, USA) at 570 nm[17]. Cell viability was determined after 3 and 7 days of cul-

ture. All samples were compared to a positive control group (RAW culture), considered as 100% cell viability.

Nitric oxide production analysis

Nitrite production was evaluated in supernatants of cell cultures by Griess reaction, with adaptations[18]. Briefly, 100 µL of cell culture supernatant was transferred to a new 96-well plate (Kasvi, China). Then, 100 µL of 1% sulfanilamide phosphoric acid solution and 2.5% of 1% naphthyl ethylenediamine phosphoric acid (1:1) was added. After 10 min, reading was performed in a microplate reader (Bio-Tek PowerWave HT, USA) at 490 nm. The amount of nitrite was calculated based on a standard curve of sodium nitrite (1.5625 µM to 200 µM)[18].

Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was performed after 7 days of incubation for the quantification of differentiated osteoclast-like cells. The tartrate-resistant acid phosphatase (TRAP) kit (Sigma-Aldrich, St. Louis, USA) was used according to the manufacturer's specifications. Osteoclast-like cells were considered as TRAP-positive cells (with red/orange TRAP staining) with more than three nuclei.

Statistical analysis

Data obtained was analyzed by the standard error of the mean for each experiment. The normality was evaluated (Kolmogorov-Smirnov test), and subsequent parametric statistical analysis was carried out by two-way analysis of variance (two-way ANOVA) for the data from MTT and NO production and one-way ANOVA for TRAP analyses. Tukey's posthoc test was applied to identify statistical differences. Analyses were considered at the 95% significance level, and statistical differences were considered when $p<0.05$. Statistical analysis was performed using GraphPad Prism 6.0 software (Instat California, USA).

Results

HDP cytotoxicity

The cytotoxicity of substances was determined by cell viability assays after 3 and 7 days of cell culture in the presence of HDPs clavanin A, clavanin MO, LL-37, $Ca(OH)_2$ and doxycycline. HDPs and $Ca(OH)_2$ were not cytotoxic to pre-osteoclasts (data not shown). However, doxycycline, at the high concentration (128 $\mu g.mL^{-1}$) reduced cell viability by 48% ($p<0.05$), after 3 days of incubation, compared to the control group (data not shown). Similar viability results were observed in osteoclast-like cells (RAW cells with rRANKL), and substances after 3 and 7 days incubation. HDPs, $Ca(OH)_2$ and doxycycline were not cytotoxic to rRANKL-stimulated cells (Figure 1). However, 128 $\mu g.mL^{-1}$ of doxycycline reduced cell viability by 42% after 3 days, compared to the control group ($p<0.05$). Indeed, HDPs were not cytotoxic and only doxycycline at 128 $\mu g.mL^{-1}$ demonstrated a cytotoxic effect on osteoclast-like cells (rRANKL-stimulated and RAW cells).

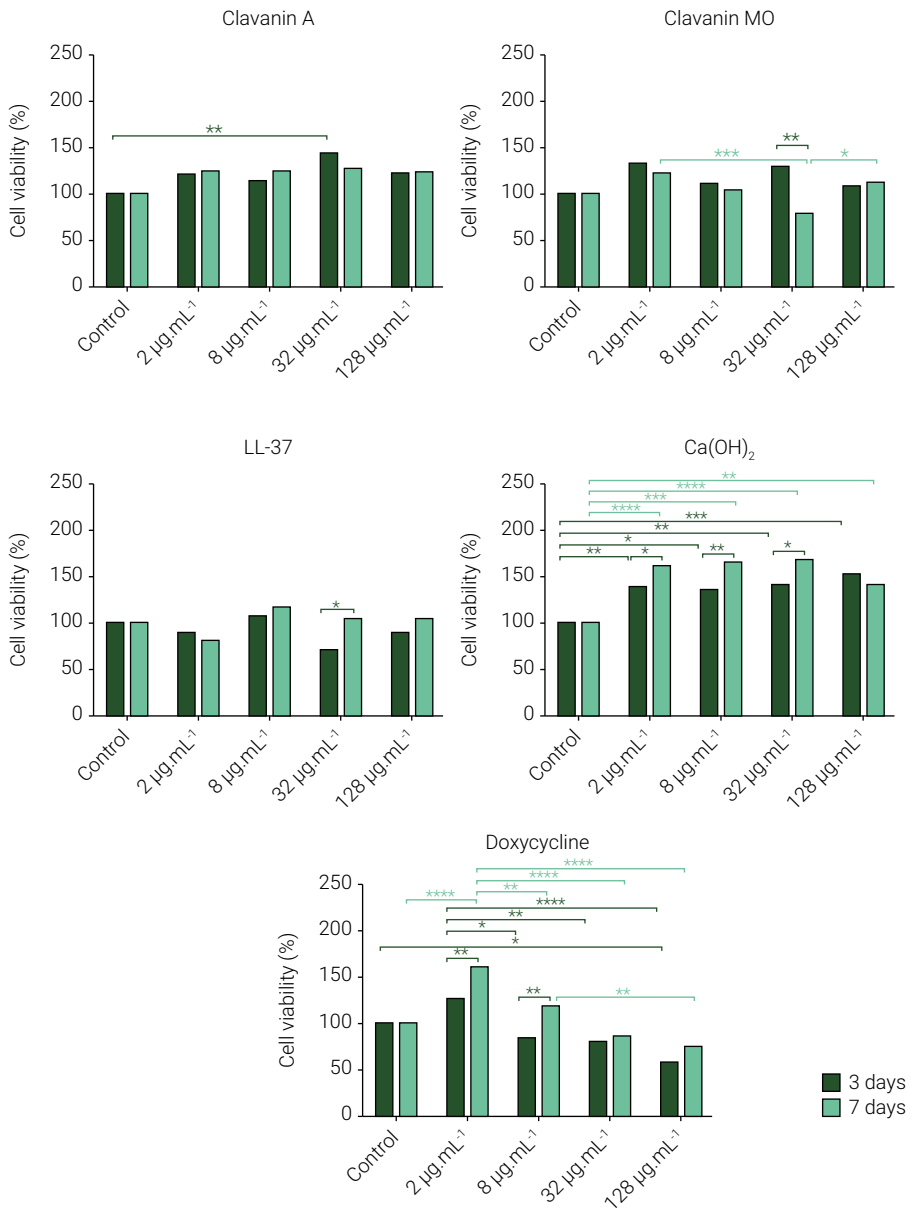

Figure 1. Clavanin A, clavanin MO, LL-37, $Ca(OH)_2$ and doxycycline cytotoxicity at 2, 8, 32 and 128 µg.mL^{-1} on 2.5x10^3 RAW cells, after 3 and 7 days, by MTT assay. Cultures were stimulated with 100 ng.mL^{-1} of rRANKL. Cell viability was represented by percentage. Control group was represented by 2.5x10^3 RAW cells stimulated with 100ng.mL^{-1} of rRANKL and considered 100% of cell viability. All experiments were done in technical and biological triplicates. Statistical differences by two-way ANOVA test and Tukey's post hoc were represented by *p<0.05, **p<0.005, ***p<0.0005 and ****p<0.0001 compared to each concentration and time tested conditions; Dark green bars represent statistical differences observed on day 3; Light green bars represent statistical differences observed on day 7.

Nitric oxide production

Cell cultures with HDPs, $Ca(OH)_2$, and doxycycline produced basal levels of NO, compared to the control group (data not shown). The rRANKL increased NO production in

RAW 264.7 cell cultures (Figure 2). After 3 days, rRANKL-stimulated cells with clavanin A downregulated NO levels at all concentrations, compared to the control group, while on the seventh day, NO levels were similar to the control group ($p<0.0001$). The down-regulation of NO production was also observed in cultures stimulated with clavanin

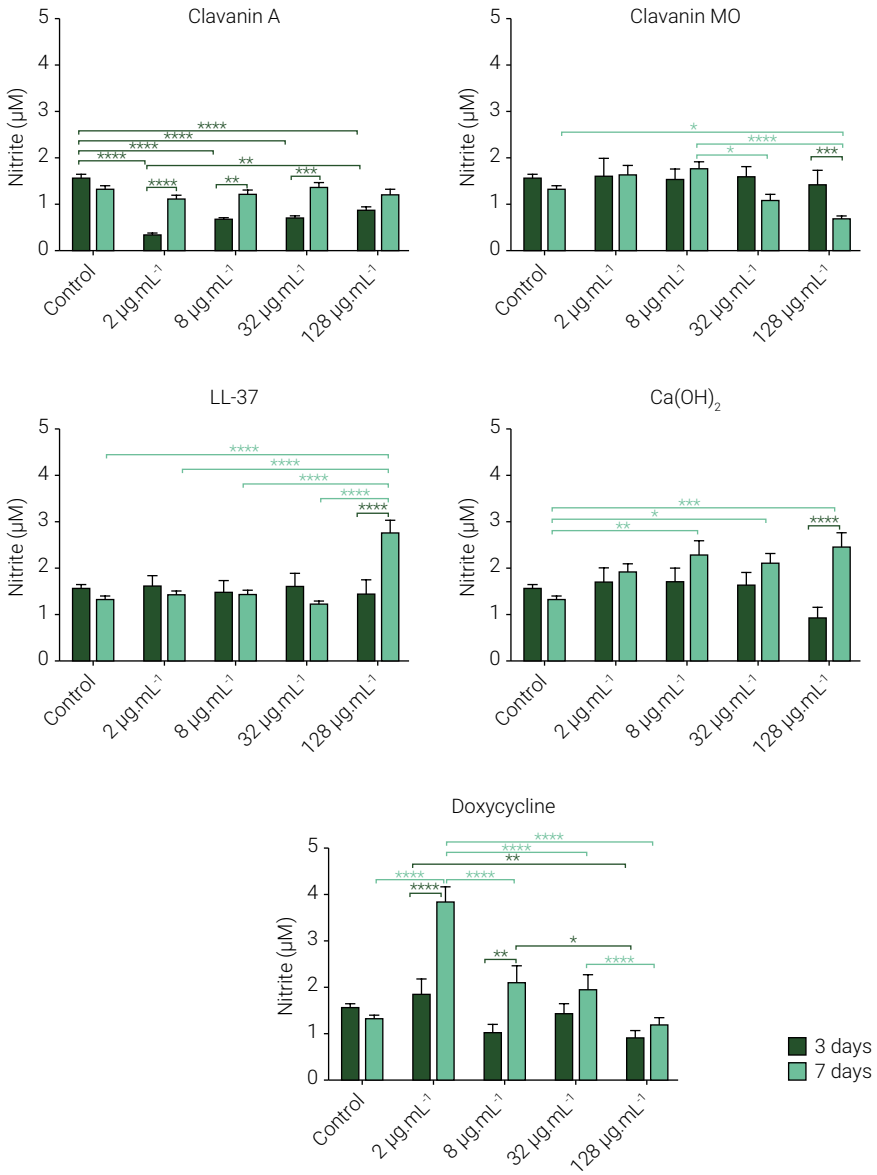

Figure 2. Nitric oxide production in the presence of clavanin A, clavanin MO, LL-37, $Ca(OH)_2$ and doxycycline at 2, 8, 32 and 128 µg.mL^{-1} on rRANKL-stimulated-RAW cells, after 3 and 7 days, as described in the method of Green et al., with adaptations. Cultures were stimulated with 100 ng.mL^{-1} of rRANKL. Control group consisted of 2.5x10^3 RAW cells stimulated with 100 ng.mL^{-1} of rRANKL. Bars represent the standard error of the mean of nitrite oxide production. All experiments were done in technical and biological triplicates. Statistical differences by two-way ANOVA test and Tukey's post hoc were represented by *$p<0.05$, **$p<0.005$, ***$p<0.0005$ and ****$p<0.0001$ compared to each concentration and time-tested conditions; Dark green bars represent statistical differences observed on day 3; Light green bars represent statistical differences observed on day 7.

MO at 128 μg.mL^{-1}, after 7 days ($p < 0.05$). However, the presence of 128 μg.mL^{-1} of LL-37 after 7 days, increased the NO levels compared to the control group ($p<0.0001$), while the others concentrations at 3 and 7 days were similar to baseline levels. $Ca(OH)_2$ was able to upregulate NO production at 8 ($p<0.005$), 32 ($p<0.05$) and 128 μg.mL^{-1} ($p<0.005$) after 7 days of cell incubation. NO levels in the presence of $Ca(OH)_2$ at other different concentrations were similar to the control group ($p<0.05$), after 3 and 7 days. The lower concentration of doxycycline up-regulated NO production after 7 days compared to all concentrations, including the control group ($p<0.0001$). Overall, reduced levels of NO were observed in some concentrations of all substances, except for doxycycline and $Ca(OH)_2$ 7 days after the test.

Number of differentiated osteoclast-like cells

RAW cell cultures stimulated with HDPs, $Ca(OH)_2$, doxycycline and rRANKL were submitted to TRAP staining after 7 days of incubation (Figure 3A-B), for quantification of differentiated osteoclast-like cells. All HDPs, $Ca(OH)_2$, and doxycycline, at all tested concentrations, were capable of reducing the differentiation of osteoclast-like cells (Figure 3B). Indeed, clavanin A reduced osteoclastogenesis in an inverse dose-dependent concentration. Clavanin A at 128 μg.mL^{-1} demonstrated the lowest number of differentiated osteoclast-like cells ($p<0.05$). Osteoclastogenesis was similarly reduced by clavanin MO at all tested concentrations ($p<0.05$). Likewise, LL-37 also downregulated osteoclastogenesis at 2, 4, 8 and 128 μg.mL$^{-1,}$ with the lowest number of osteoclast-like cells in 2 and 4 μg.mL^{-1} ($p<0.05$). $Ca(OH)_2$ was most effective in reducing osteoclast-like cells at 8 μg.mL^{-1} ($p<0.05$). However, 2 μg.mL^{-1} of $Ca(OH)_2$ showed the highest number of differentiated osteoclast-like cells ($p < 0.05$). Meanwhile, doxycycline exhibited a gradual reduction in osteoclastogenesis, and 128 μg.mL^{-1} stimulated cells demonstrated the lowest number of differentiated osteoclast-like cells.

Based on the number of osteoclast-like cells differentiated by rRANKL-stimulated RAW cell culture, the concentration of 8 μg.mL^{-1} was the lowest common concentration for HDPs, $Ca(OH)_2$, and doxycycline, capable of reducing differentiation in osteoclast-like cells. Therefore, the best results were exhibited by LL-37 and $Ca(OH)_2$. LL-37 showed approximately 67% fewer osteoclasts than clavanin A, clavanin MO and doxycycline, while $Ca(OH)_2$ showed 59% fewer osteoclasts compared to clavanins and doxycycline. $Ca(OH)_2$ and LL-37 demonstrated better osteoclastogenesis downregulation, compared to the same concentration of clavanin A, clavanin MO and doxycycline ($p<0.0001$). Therefore, among the tested HDPs, LL-37 presented the best ability to reduce the number of osteoclasts *in vitro* ($p<0.05$).

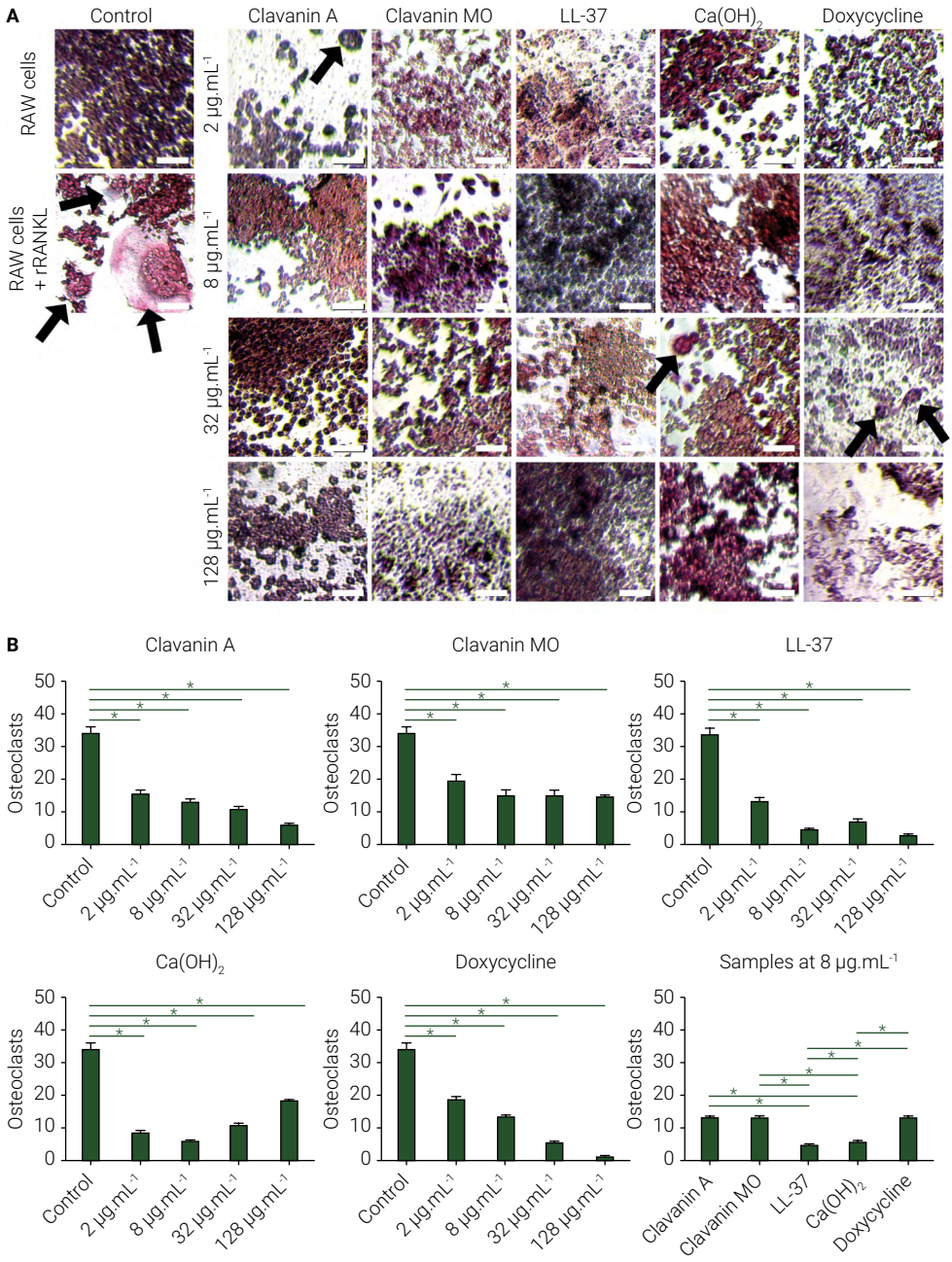

Figure 3. Representative photos of TRAP positive stained cells (A) and number of multinucleated osteoclast-like cells (B) induced by $2.5x10^3$ RAW cell and $2.5x10^3$ RAW cells stimulated with 100 $ng.mL^{-1}$ rRANKL (Control). Cultures were rRANKL-stimulated and tested with 2, 8, 32, and 128 $\mu g.mL^{-1}$ of clavanin A, clavanin MO, LL-37, $Ca(OH)_2$ and doxycycline, after 7 days. Scale bar: 50 µM. Each well was completely checked, and osteoclasts were counted at 20x magnification. Black arrows show osteoclast-like cells with more than 3 nuclei. Statistical differences by one-way ANOVA test and Tukey's post hoc were represented by *p<0.001 compared to control in each tested condition. Statistical differences of comparative analysis of the lowest common concentration, with greater reduction in osteoclast-like cell differentiation, of all tested materials: 8$\mu g.mL^{-1}$ of clavanin A, clavanin MO, LL-37, $Ca(OH)_2$ and doxycycline (samples at 8$\mu g.mL^{-1}$) were represented by *p<0.0001. Number of osteoclast-like cells was represented as the standard error of the mean. All experiments were done in technical and biological triplicates.

Discussion

Although periodontal and endodontic therapies are highly effective, new substances can improve outcome expectations. Antimicrobial, immunomodulatory, and reparative activity could be better achieved by new therapies and biologic substances[19]. Indeed, antimicrobial resistance is also a current limitation for both therapies[6]. The present study demonstrated the *in vitro* potential of HDPs clavanins A and MO in an osteoclastogenesis model. Results of cellular NO production, cytotoxicity, and the effects of HDPs on rRANKL-mediated osteoclastogenesis were compared to $Ca(OH)_2$ and doxycycline, widely used medications in endodontic[6] and periodontal[5] areas, respectively.

Study related to clavanin A has demonstrated different activities regarding this peptide, including important points for dentistry, such as antibiofilm and antimicrobial activity. HDP clavanin A showed antibiofilm activity against fungal biofilms when used to coat an amniotic membrane, which is frequently used in ophthalmologic surgery for rapid ocular surface reconstruction[20]. HDPs clavanin A, clavanin MO and LL-37 did not show any degree of cytotoxicity to RAW cells. A previous study using clavanin A also showed no cytotoxicity against mammalian cells (L929) with low concentrations[9]. Moreover, another study showed that 128 $\mu g.mL^{-1}$ of clavanin MO did not demonstrate cytotoxicity compared to the other antimicrobial agents, with or without additional stimulation. LL-37 increased cell viability on RAW cells, and $Ca(OH)_2$ did not interfere with cell viability at the same concentration. Besides, after 6 h of incubation, clavanins alone reduced cell viability[14].

NO regulates bone resorption through the regulation of the synthesis of OPG/RANKL in bone marrow cells[21], although other factors, including cytokines, are also involved. Our results demonstrated that HDPs downregulated NO production with or without the stimulation of rRANKL. Accordingly, clavanin A demonstrated a significant reduction in the number of osteoclast-like cells in a dose-dependent manner. Clavanin MO also reduced the number of differentiated osteoclast-like cells. Similarly, LL-37 at 8 and 128 $\mu g.mL^{-1}$ demonstrated the best inhibition activity. According to previous results, HDPs can also modulate inflammatory mediators that contribute to the bone resorption activation process, such as TNF-α, IL-6 and IL-1α, and NO production[14].

Different substances already used in clinical practice were also evaluated in this study. $Ca(OH)_2$ did not show cytotoxicity, and doxycycline demonstrated a toxic effect on cells at high concentration and increased cell viability at low concentration. Accordingly, a study evaluated the effects of a sub-antimicrobial dose of doxycycline (SDD) on ligature-induced periodontitis in spontaneously hypertensive rats. It concluded that SDD therapy exerted a systemic modulating effect on inflammation, with reduced periodontal tissue destruction in hypertensive rats[22].

$Ca(OH)_2$ presented similar NO results compared to HDPs, and doxycycline presented an increase in NO levels, especially at 2 $\mu g.mL^{-1}$. In the osteoclastogenesis process, both tested drugs decreased the number of osteoclast-like cells in the presence of all concentrations tested. These facts suggest that although NO is strongly associated with osteoclast differentiation, this is not the only factor involved in the osteoclasto-

genesis process[23]. Also, doxycycline might have another mechanism for downregulating the osteoclastogenesis pathway[23].

However, these results are in agreement with previous results that suggest the inhibition of osteoclastogenesis in RAW cells in the presence of $Ca(OH)_2$[23]. Other studies suggest that the alkaline pH of $Ca(OH)_2$ can neutralize the lactic acid secreted by osteoclasts and may help prevent the destruction of mineralized tissue[24].

In summary, this study aims to initiate the assessment of the biotechnological potential of HDPs clavanin A and MO in the oral osteoimmunological context and their capability to reduce *in vitro* osteoclastogenesis, compared to LL-37 (HDP control), $Ca(OH)_2$ (used in the endodontic treatment) and doxycycline (used in the periodontal treatment). We highlighted the results observed in the presence of 8 $\mu g.mL^{-1}$ of LL-37 and $Ca(OH)_2$, thus considering the use of these peptides as a possible product for endodontic and periodontal applications, in order to reduce the osteoclastogenesis process. On the other hand, $Ca(OH)_2$ shows low production costs when compared to LL-37. This HDP presents a relatively long sequence of amino acids, which raises its cost for synthesis, and it would probably only be indicated for restricted cases. Indeed, because of its immunomodulatory benefits and its biocompatibility, by being a peptide present in the oral cavity, LL-37 presents itself as a good candidate for dentistry use. When LL-37 results were compared to the doxycycline, the HDP demonstrated better efficiency in osteoclastogenesis downregulation at low concentrations, thus showing an even greater potential in the context of periodontal bone loss.

Despite the benefits highlighted in these data, *in vitro* results should be interpreted with caution and other *in vivo* studies are necessary to evaluate the potential of this biomolecule for clinical use. Other important points for future investigations should be focused on the large-scale expression of this peptide (lowering its cost), and the analysis of its integrity through various oral conditions, such as temperature changes, pH, and presence of lytic enzymes. In addition, other parameters should be evaluated, such as the peptides' mechanism of action in the osteoclastogenesis process, in order to enhance the knowledge on these potential products indicated for bone resorption processes, present in the periradicular area and periodontitis.

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