Complex osteoclastogenic inductive effects of nicotine over hydroxyapatite

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Cigarette smoke is associated to pathological weakening of bone tissue, being considered an important playmaker in conditions such as osteoporosis and periodontal bone loss. In addition, it is also associated with an increased risk of failure in bone regeneration strategies. The present work aimed to characterize the effects of nicotine on human osteoclastogenesis over a hydroxyapatite substrate. Osteoclast precursors were maintained in the absence or presence of the osteoclastogenesis enhancers M-CSF and RANKL, and were further treated with nicotine levels representative of the concentrations observed in the plasma and saliva of smokers. It was observed that nicotine at low concentrations elicit an increase in osteoclast differentiation, but only in the presence of M-CSF and RANKL it was also able to significantly increase the resorbing ability of osteoclasts. A slight downregulation of NFkB pathway and an increase in the production of TNF-α and, particularly PGE2, were involved in the observed effects of nicotine. At high concentrations, nicotine revealed cytotoxic effects, causing a decrease in cell density. In conclusion, nicotine at levels found in the plasma of the smokers, has the ability to act directly on osteoclast precursors, inducing its osteoclastogenic differentiation. The stimulatory behavior appears to be dependent on the stage of osteoclastic differentiation of the precursor cells, which means, in the absence of M-CSF and RANKL, it only favors the initial stages of osteoclast differentiation, while in the presence of the growth factors, a significant increase in their resorbing ability is also achieved.

KEYWORDS
hydroxyapatite, nicotine, osteoclastogenesis

1 | INTRODUCTION

Osteoclasts are multinucleated cells specialized in bone resorption. They are originated from the fusion and further differentiation of mononuclear precursors that belong to the CD14+ monocyte/macrophage lineage (Datta, Ng, Walker, Tuck, & Varanasi, 2008; Vaananen & Laitala-Leinonen, 2008). Osteoclastogenesis is a complex process that requires an intricate network of cellular crosstalks involving many signaling molecules, including the main activators M-CSF and RANKL (Boyle, Simonet, & Lacey, 2003; Datta et al., 2008; Zhao, Shao, Chen, & Li, 2007). Once committed to osteoclast development, precursor cells pass through multiple steps of intracellular rearrangements that ultimately lead to the generation of mature, bone-resorbing osteoclasts. One important mediator of the final events of osteoclast activation is the tyrosine-kinase c-src (Boyle et al., 2003; Zhao et al., 2007). Once activated, they start to transport H^+ to the extracellular bone surface, eroding the bone hydroxyapatite crystals and, at the same time, they secrete lytic enzymes, especially cathepsin K, that degrade the organic components of bone matrix (Boyle et al., 2003; Vaananen & Laitala-Leinonen, 2008).

Cigarette smoke has been associated with several bone disturbances like osteoporosis and periodontal bone loss (Ahmed & Mohammed, 2002; Baheiraei, Pocock, Eisman, Nguyen, & Nguyen, 2005; Balatsouka, Gotfredsen, Lindh, & Berglundh, 2005; Law & Hackshaw, 1997). These conditions may be attributed partly to the known inflammatory effects of tobacco smoke, although a direct effect on bone cells may also occur. Tobacco smoke contains more than a thousand of potentially toxic substances, but nicotine has been suggested as a key molecule toward the understanding of the negative effects of smoking on bone tissue (Pereira, Carvalho, Peres, & Fernandes, 2009; Seiichi et al., 2010). It is known that nicotine negatively affects blood flow to the bone and surrounding tissues,

which difficult the healing processes and can also lead to problems related with the delivery of nutrients to bone cells (Jeffrey, John, Kun, Peiman, & Dave, 1999). As a consequence, nicotine can cause bone graft morbidity and impaired bone healing, and also compromise the success of bone regeneration strategies (Law & Hackshaw, 1997). In addition, a direct effect of nicotine on bone cells may also contribute to the observed deleterious effects of smoking in bone tissue (Balatsouka et al., 2005).

Several studies have shown that nicotine modulates osteoblast activity in a dose-dependent way (Pereira, Carvalho, Peres, Gutierres, & Fernandes, 2008; Pereira et al., 2009). Overall, and despite the variability of the available information, due to the different experimental protocols regarding nicotine levels, exposure conditions and cell culture models, results about the effect of nicotine on osteoblastic cells show that this molecule may play a significant role in the modulation of bone metabolic activities, suggesting a potential impact in the overall bone health (Pereira et al., 2008, 2009; Seiichi et al., 2010; Yuhara et al., 1999). By contrast, the effects of nicotine on osteoclastic cells, the other key player in bone remodeling and regeneration, are scarcely documented. The few published studies were not conducted with human cells, instead it was used mouse and porcine cells (Henemyre et al., 2003; Tanaka et al., 2013; Yuhara et al., 1999). It was reported that co-cultures of mouse bone marrow and ST2 cells displayed an inhibition of osteoclastogenesis caused by nicotine (Yuhara et al., 1999). In another study, nicotine stimulated the resorbing activity of porcine osteoclastic cells (Henemyre et al., 2003), whereas, in a more recent work, nicotine reduced the resorbing ability of RAW264.7 murine cells (Tanaka et al., 2013). The different protocols and the origin of the analyzed cells might explain the apparent observed contradictions, and reinforces the need to address this issue in more detail and in a more representative model, as in human primary osteoclastic cells.

Since the inorganic matrix of bone tissue is mainly comprised by combined calcium and phosphate ions, calcium phosphate biomaterials display good biocompatibility and osteoconductivity, having an established clinical relevance in bone related applications (Dimitriou, Jones, McGonagle, & Giannoudis, 2011). As such, synthetic hydroxyapatite (HA), has been successfully used for bone replacement/regeneration over the years (Rumpel et al., 2006). Upon material implantation, osteoclastic cells are key players in the cellular events occurring at the bone/HA interface. Together with the osteoblastic cells, they are involved in the bone regeneration process occurring at early implantation stages, due to their high affinity for calcium phosphates surfaces, but also during the subsequent local bone remodeling responsible for the maintenance of the life-long mechanical and biological properties of the regenerated bone (Datta et al., 2008). Thus, understanding the effects of exogenous molecules, such as nicotine, in the bone cells/HA interface is a critical issue for a long-term successful implantation. However, this has not yet been documented regarding the osteoclastic behavior.

In this context, the aim of this work is to provide a detailed information on the dose- and time-dependent effects of nicotine in the differentiation and functional activity of human osteoclastic cells occurring over an HA substrate. For that, human osteoclastic precursors were cultured over HA in the absence or the presence of osteoclastogenic promoters (M-MSF and RANKL), for the evaluation of the nicotine effects in unstimulated precursors and precursor cells actively engaged in a differentiation process. Cell cultures were exposed to a range of plasmatic and salivary nicotine concentrations and were assessed for several osteoclast-related parameters. The involvement of some signaling pathways on the cellular response was also addressed.

2 | MATERIALS AND METHODS

2.1 | Preparation and characterization of hydroxyapatite disks

Commercial Hydroxyapatite (HA) (Plasma Biotal Limited, Buxton, United Kingdom) was used to prepare disks with 16 mm of diameter, as described before (Costa-Rodrigues, Fernandes, Lopes, & Fernandes, 2012). The discs were obtained using 1 g of dry powder under uniaxial compression stress of 140 kg/cm^2 (Mestra snow P3). The sintering temperature used was of 1,300°C, with a 15 min plateau and applying a heating rate of 20°C/min. The sintering cycle was completed with a cooling process inside the furnace (Termolab/Eurotherm 2408). The mass of the HA discs was assessed by using an analytical balance (Metler AG285). After the sintering process, the disks were polished with a Dual Disc Polishing Machine (Stuers Rotopol-1), with waterproof sandpaper (# 320, # 500, and # 1000).

2.1.1 | X-ray diffraction (XRD) analysis

HA disks were ground and the resulting fine powders were analyzed by an X-ray diffraction Rigaku Dmax-III-VC, using a CU-Kα radiation (Kα = 1.54056 Å). Data were acquired for 2θ values between 4 and 80° at intervals of 0.02°/s.

2.1.2 | Scanning electron microscopy (SEM)

Analyses were performed using a SEM microscope FEI Quanta 400FEG/EDAX Genesis X4M under vacuum conditions. HA disks were coated with a thin film of gold using a sputter coater (SPI-Module) in an atmosphere of argon before analysis.

2.2 | Osteoclastic cell cultures

Human peripheral blood mononuclear cells (PBMC) were used to establish the osteoclastic cell cultures. PBMC were isolated from the blood of healthy donors with 25–35 years old, after informed consent, as described previously (Costa-Rodrigues et al., 2012). Briefly, blood was diluted with PBS (1:1), supplemented with 2 mM EDTA and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400*g* for 30 min, PBMC were recovered and washed twice with PBS+ 2 mM EDTA. Typically, around 70×10^6 PBMC were obtained for each 100 ml of processed blood. PBMC cells were counted with a cytometer (Celltac MEK-5103).

PBMC (1.5×10^6 cells/cm^2) were cultured in α-MEM supplemented with 30% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine. PBMC were cultured for 21 days in the absence (base medium, BM) or presence (M+R) of the osteoclastogenic inducers M-CSF (25 ng/ml) and RANKL (40 ng/ml) (Costa-Rodrigues et al., 2012). When indicated, 0.5 μg/ml of isotype control or anti-RANKL antibodies (Abcam, Cambridge, United Kingdom) were included in the culture media. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C, and culture medium was replaced once a week.

2.2.1 | Exposure to nicotine

As a preliminary experiment, unstimulated and stimulated PBMC were exposed to a wide concentration range of nicotine, that is, 1.28 ng/ml to 500 μg/ml. Nicotine was added 24 hr after cell plating, and it was present during the 21-day culture time, being renewed at each medium change. Cultures were assessed for total protein content and TRAP activity.

Based on this experiment, PBMC cultured over HA surface were exposed to 1.28 ng/ml to 100 μg/ml nicotine, in conditions similar to those described above, and were characterized for total protein content and TRAP activity; they were also observed by confocal laser scanning microscopy (for the presence of actin rings and vitronectin and calcitonin receptors) and by scanning electron microscopy. Cultures treated with 1.28 and 32 ng/ml nicotine and 4 μg/ml, representative concentrations of statistically significant effects on TRAP activity, were characterized for the expression of several osteoclastic genes. Also, PBMC cultures exposed to 32 ng/ml nicotine, which it was the lowest tested concentration that elicited a significant effect on osteoclastogenesis, both in BM and M+R conditions, were also characterized for HA resorbing ability and for the involvement of several intracellular pathways and RANKL signaling. The culture medium was also analyzed for the levels of TNF-α and PGE2. Cultures were assessed at days 14 and 21 as described below.

2.3 | Osteoclastogenic cell response

2.3.1 | Protein quantification

Quantification of total protein content was performed by Bradford's method (Bradford, 1976), using bovine serum albumin as a standard. Shortly, cell cultures were washed with PBS, solubilized with 0.1 M NaOH and treated with Coomassie® Protein Assay Reagent (Fluka). After incubation for 2 min at room temperature, the 595 nm absorbance was determined in an ELISA plate reader (Synergy HT, Biotek, Winooski, VT).

2.3.2 | TRAP activity

TRAP activity was assayed by *para*-nitrophenilphosphate (*p*NPP) hydrolysis method. Cell cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100. Cellular extracts were incubated with 12.5 mM *p*NPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 hr at 37°C. After addition of 5 M NaOH, the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). TRAP activity was normalized with total protein content, quantified by Bradford's method, and results were expressed as $nmol/min.\mu g_{protein}^{-1}$.

2.3.3 | Apoptosis quantification

Apoptosis was evaluated by means of caspase-3 activity quantification. PBMC cultures, at days 14 and 21, were washed twice in PBS and assessed for caspase 3 activity with EnzCheck® Caspase-3 Assay Kit #2 (Molecular Probes, Eugene), according to manufacturer's instructions. Fluorescence was measured at 496/520 nm (excitation/emission) in an ELISA plate reader (Synergy HT, Biotek). Results obtained in each experimental condition were normalized by the value obtained in the corresponding control (absence of nicotine) and presented as a percentage.

2.3.4 | Cells with actin rings and expressing vitronectin and calcitonin receptors

Cell cultures were fixed (3.7% (v/v) para-formaldehyde, 15 min) permeabilized (0.1% (v/v) Triton X-100, 5 min) and stained for actin with 5 U/ml Alexa Fluor1 647-Phalloidin (Invitrogen, Carlsbad, CA), and for vitronectin and calcitonin receptors (VNR and CTR) with 50 mg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Detection of IgGs anti-VNR and IgGs anti-CTR was performed with 2 mg/ml Alexa Fluor1 488-Goat anti-mouse IgGs. Cells were observed under confocal laser scanning microscopy (CLSM).

2.3.5 | Quantitative real-time PCR analysis

PBMC cultures were analyzed by quantitative real-time PCR (qPCR) for the expression of the housekeeping genes beta-glucuronidase (GUSB) and proteasome subunit beta type-6 (PSMB6), and the osteoclast-related genes c-myc, c-src, TRAP, CATK, and CA2 (Zhao et al., 2007). For that, RNA was isolated with RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reverse transcription cDNA synthesis was performed with DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) and random hexamers according to the manufacturer's instructions. Then, cDNA samples (2 ng) were amplified in with a DyNAmo Flash SYBR green qPCR kit (Finnzymes) on a Rotor-Gene thermocycler (Qiagen). The annealing temperature used was 55°C and the extension time was 15 s. The primers used are listed in Table 1. The values obtained were normalized with the results obtained for the two tested housekeeping genes.

2.3.6 | Quantification of Ca^{2+} levels in the culture medium

Ca^{2+} levels in the medium, collected at days 14 and 21, were analyzed by using the the CalciFluor™ Assay, according to manufacturer's instructions. The fluorescence of the samples was measured in a fluoremeter with plate reader (Synergy HT, Biotek) at 530/590 nm (excitation/emission). The concentration of Ca^{2+} in the culture medium was corrected taking into account the corresponding values observed in the culture medium from HA disks incubated without cells and in the culture medium from PBMC cultures performed in standard tissue culture plates.

TABLE 1 Primers used on qPCR analysis of PBMC cultures

Gene	5′ Primer	3′ Primer
GUSB	5′-TGCAGCGGTCTGTACTTCTG-3′	5′-CCTTGACAGAGATCTGGTAATTCA-3′
PSMB6	5′-GCCGGCTACCTTACTACCTG-3′	5′-AAACTGCACGGCCATGATA-3′
TRAP	5′-ACCATGACCACCTTGGCAATGTCTC-3′	5′-ATAGTGGAAGCGCAGATAGCCGTT-3′
CATK	5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′	5′-CTTGCATCAATGGCCACAGAGACAG-3′
CA2	5′-GGACCTGAGCACTGGCATAAGGACT-3′	5′-AAGGAGGCCACGAGGATCGAAGTT-3′
c-myc	5′-TACCCTCTCAACGACAGCAG-3′	5′-TCTTGACATTCTCCTCGGTG-3′
c-src	5′-AAGCTGTTCGGAGGCTTCAA-3′	5′-TTGGAGTAGTAGGCCACCAG-3′

2.3.7 | Scanning electron microscopy (SEM)

Cells were fixed (1.5% glutaraldehyde in 0.14 M sodium cacodylate, 10 min), dehydrated in graded series of ethanol solutions and critical-point dried. Specimens were mounted onto aluminium supports using Araldite™ and then sputter-coated with palladium–gold and observed in a Joel JSM 35C scanning electron microscope equipped with an X-ray energy dispersive spectroscopy voyager XRMA System (Noran Instruments, Pacifica, CA). Total resorbed area was quantified. For that, cell layers were bleached, 10 micrographs were randomly chosen for each experimental condition and the resorption lacunae were identified. The corresponding areas were calculated with ImageJ 1.41 software. Results were expressed as the percentage of the total area of the micrographs that were identified as resorption lacunae.

2.3.8 | Intracellular signaling mechanisms

PBMC cultures were treated with specific inhibitors of some osteoclastogenic-related signaling pathways, namely: U0126, a MEK signaling pathway inhibitor, that was tested at 1 and 10 μM, because of the differing osteoclastogenic effects observed with low concentrations of this molecule (Costa-Rodrigues, Teixeira, & Fernandes, 2011; Hotokezaka et al., 2002; Kim et al., 2007; Yang et al., 2008); PDTC, a NFkB signaling pathway inhibitor, was used at 10 and 100 μM, because the lower concentration has been previously described as the IC50 on rat osteoclastic differentiation (Hall, Schaeublin, Jeker, Fuller, & Chambers, 1995); indomethacin (1 μM) a PGE2 synthesis blocker, was also included in the study, as PGE2 is reported to be associated with an increase on osteoclastic development (Kawashima, Fujikawa, Itonaga, Takita, & Tsumura, 2009; Kellinsalmi et al., 2007). PBMC cultures were analyzed for TRAP activity.

2.3.9 | TNF-α and PGE2 quantification on culture media

Culture medium from PBMC cultures was analyzed for TNF-α and PGE2 levels, using the TNF alpha Human ELISA Kit (Abcam) and Prostaglandin E2 ELISA Kit (Abcam), respectively, following manufacturer's instructions. Absorbance of the samples was measured at 450 nm (TNF-α) and 405 nm (PGE2) in an ELISA plate reader (Synergy HT, Biotek). Results were expressed as pg/ml.

2.4 | Statistical analysis

Data presented in this work are the means of three separate experiments performed with cells from four different blood donors. Statistical differences were assessed using an unpaired Student's *t*-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean ± standard. For values of $p \leq 0.05$, differences were considered statistically significant.

3 | RESULTS

3.1 | HA disks

HA disks were analyzed by SEM and XRD techniques (Figure 1). HA displayed a regular surface with widespread small cavities (<10 μm) (Figure 1a). XRD spectrum (Figure 1b), showed that the disks were composed exclusively by HA, with a greater crystallinity between 30° and 35° 2θ.

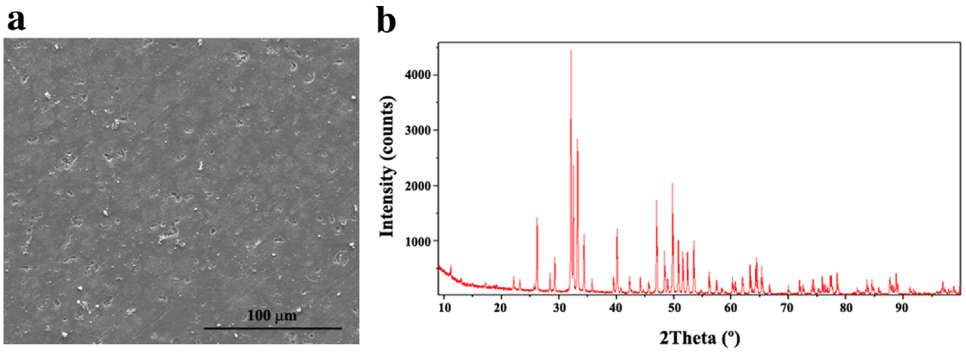

FIGURE 1 HA disks characterization by SEM (a) and XRD (b)

3.2 | Dose-response effect of nicotine on TRAP activity by PBMC cultures

Control PBMC cultures performed in base medium (BM) presented low protein content, and nicotine at concentrations higher than 20 μg/ml elicited a dose-dependent decrease. Regarding TRAP activity, PBMC cultures maintained in BM revealed low enzyme activity, and exposure to nicotine caused an inductive effect at concentrations of 32 to 800 ng/ml (39–97% at day 21), and a dose-dependent decrease at higher levels. Caspase-3 activity was significantly increased, either at day 14 or 21, in the presence of nicotine concentrations ≥20 μg/ml (18–51% or 20–62%, respectively). PBMC cultures supplemented with M-CSF and RANKL (M+R) presented a significantly higher protein content and TRAP activity. Nicotine, at concentrations >4 μg/ml caused a dose-dependent decrease in total protein content. Moreover, nicotine at 6.4 and 32 ng/ml increased TRAP activity at day 21 (~30%); exposure to levels ≥0.8 μg/ml resulted in dose-dependent negative effects. Apoptosis was stimulated by nicotine at concentrations ≥4 μg/ml, being 26–66% higher than the control at day 14, and 31–70% at day 21. Results are summarized in Figure 2.

3.3 | Effect of nicotine on osteoclastogenesis over a HA substrate

3.3.1 | TRAP activity

The effect of nicotine on PBMC cultured over the HA surface was similar to that observed in standard tissue culture plates (Figure 3a), that is, total protein content was only affected (negatively) by high nicotine concentrations (Figure 3a), and there was an inductive effect on TRAP activity at ~32 ng/ml nicotine, and an inhibitory effect at higher levels (Figure 3b).

3.3.2 | Presence of cells with actin rings and expressing VNR and CTR

PBMC displayed well-defined actin rings and VNR and CTR, as observed by CLSM. Overall, both in control conditions and in the presence of nicotine, the amount of osteoclastic cells was in line with the results obtained for TRAP activity. Representative micrographs of PBMC cultures, exposed to 32 ng/ml nicotine, are depicted in Figure 3c.

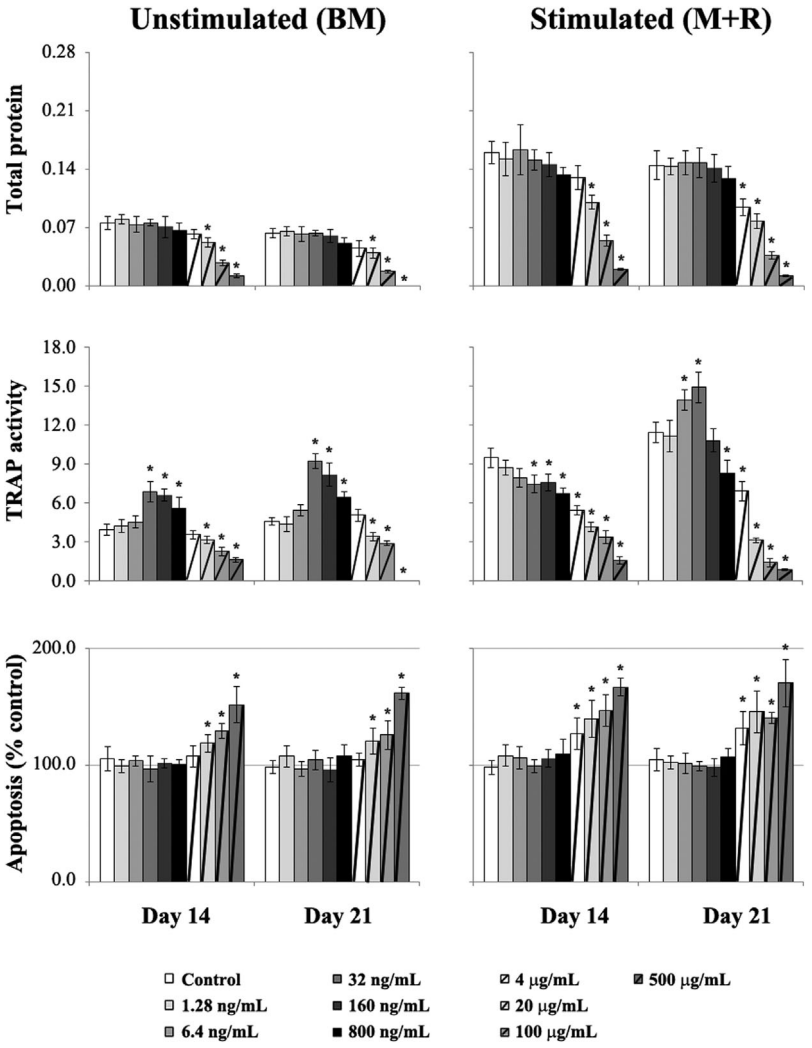

FIGURE 2 Dose-response effects of nicotine on human osteoclastic cells. Total protein content, TRAP activity and apoptosis were measured in unstimulated (base medium, BM) and stimulated (M-CSF and RANKL, M+R) PBMC cultures. *Significantly different from the control

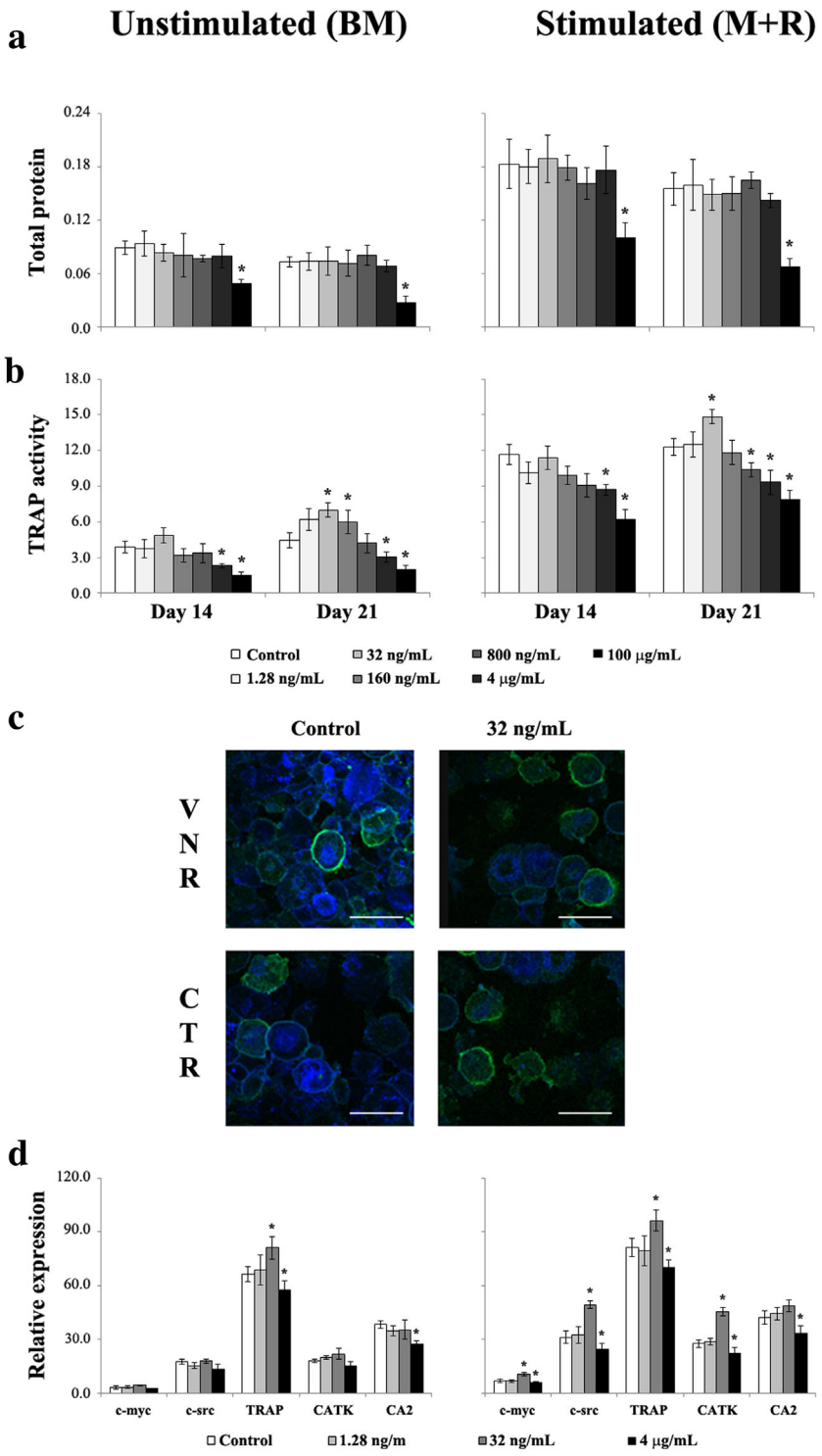

FIGURE 3 Osteoclastic cell behavior over HA disks. Unstimulated (base medium, BM) and stimulated (M-CSF and RANKL, M+R) PBMC cultures were exposed to nicotine and analyzed for total protein content (a), TRAP activity (b), presence of cells with actin rings (blue) and expressing VNR and CTR (green) observed by CLSM (c), and expression of osteoclast-related genes by qPCR (d). *Significantly different from the control

3.3.3 | Expression of osteoclast-related genes

PBMC cultures were assessed by qPCR for the expression of c-myc, c-src, TRAP, CATK, and CA2 (Figure 3d). In BM, nicotine at 1.28 ng/ml did not significantly affect gene expression; at 32 ng/ml it only stimulated the expression of the gene coding for TRAP, while the expression of the remaining genes was not affected (~23%); finally, at 4 μg/ml it elicited a decrease in TRAP and CA2 gene expression (~13% and ~29%, respectively). Compared to the control, cell cultures performed in M+R and treated with 32 ng/ml nicotine, revealed a higher gene expression of all tested genes, except CA2; the maximum increase was observed for c-src and CATK (~57 and 64%,

respectively). On the other hand, supplementation with 4 μg/ml nicotine elicited a significant decrease (~13–22%) in the expression of all the tested genes.

3.3.4 | Levels of Ca^{2+} released into culture media and total resorbed area

In control PBMC cultures performed in base medium, the amount of Ca^{2+} present in culture medium was low, but it increased significantly in the cultures supplemented with M-CSF and RANKL. Nicotine, at 32 ng/ml, did not significantly affect the concentration of Ca^{2+} in the culture medium collected from the cultures performed in base medium. However, in stimulated PBMC cultures, the presence of 32 ng/ml nicotine greatly increased Ca^{2+} levels in the medium (~60% at day 21). Results are presented in Figure 4a. Regarding total resorbed area, in BM conditions it was not observed the formation of resorption lacunae, neither in the absence or presence of nicotine. In PBMC cultures maintained in M+R media, nicotine was able to promote an increase in cell response (~32% at day 21). Results are shown in Figure 4b.

3.3.5 | Scanning electron microscopy

Figure 4c shows representative SEM images of control unstimulated and stimulated PBMC cultures and stimulated PBMC cultures exposed to 32 ng/ml nicotine. Osteoclastic cells were randomly distributed on the HA surface, in the three experimental conditions. Unstimulated control cultures showed little evidence of resorption activity. By contrast, osteoclastic cells associated with resorption lacunae were well visible on control stimulated cultures. The presence of nicotine in these cultures greatly increased the resorbing activity, evidenced by the formation of resorption lacunae on the HA surface.

3.3.6 | Signaling pathways

PBMC cultures, exposed to 32 ng/ml nicotine, were assessed for TRAP activity in the presence of several signaling pathway inhibitors (Figure 5). In control PBMC cultures (unstimulated and stimulated), TRAP activity was partially or totally inhibited in the presence of, respectively, 1 and 10 μM U0126 (MEK inhibitor). PDTC (NFkB inhibitor), at 10 and 100 μM, completely abolished TRAP synthesis. Indomethacin (which blocks PGE2 synthesis) did not cause any significant effect on the enzyme activity. In the cultures exposed to nicotine, some TRAP activity was detected in the presence of 10 μM U0126 and 10 μM PDTC, namely in stimulated cultures. In addition, indomethacin elicited a significant decrease on TRAP activity, only in cell cultures performed in the presence of M-CSF and RANKL.

3.3.7 | Contribution of RANKL signaling to the effect of nicotine

PBMC cultures exposed to 32 ng/ml nicotine were treated with antibodies anti-RANKL and were assessed for TRAP activity (Figure 6a). Either in BM or M+R conditions, control PBMC cultures revealed an almost total inhibition of TRAP activity (>95%). Comparatively, in the presence of nicotine, the inhibition was about 79% (BM) or 47% (M+R).

3.3.8 | Levels of TNF-α and PGE2 released into the culture medium

Culture medium from PBMC cultures was assessed for TNF-α and PGE2 levels (Figure 6b). In BM, nicotine elicited an increase in the production of TNF-α (51%) but did not affect PGE2 synthesis, while in

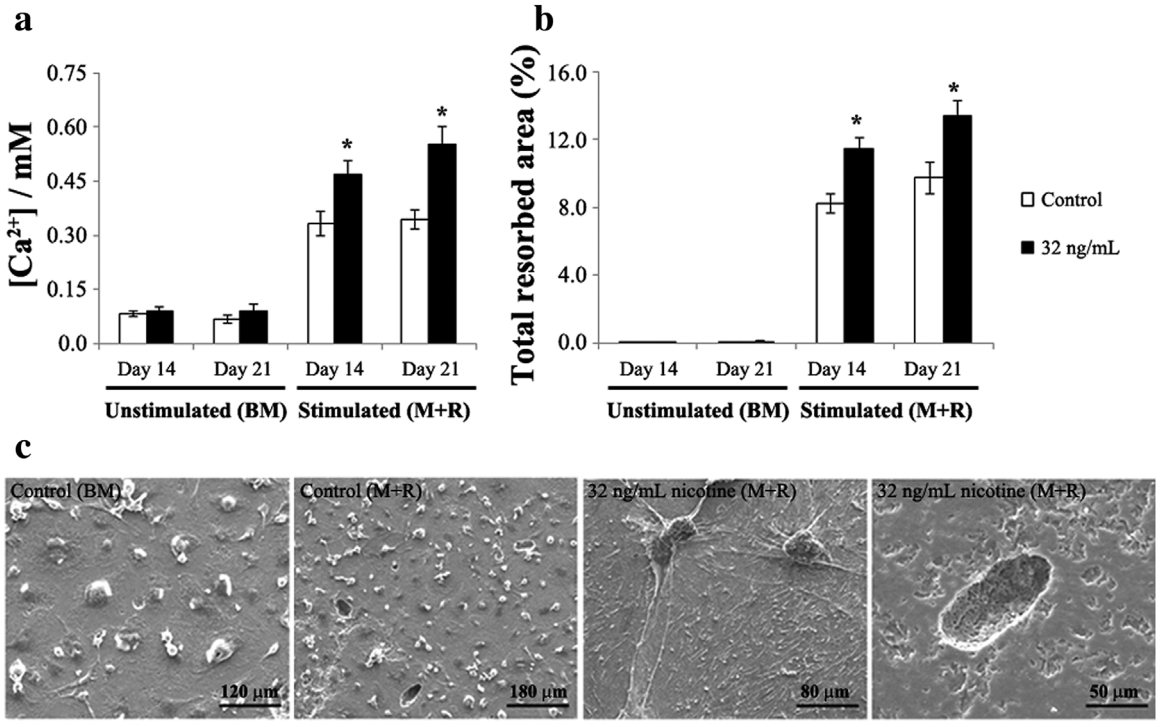

FIGURE 4 Quantification of (a) Ca^{2+} released to culture media, (b) total resorbed area and (c) SEM visualization of unstimulated (BM) and stimulated (M+R) PBMC cultures exposed to nicotine and performed over HA disks. *Significantly different from the control

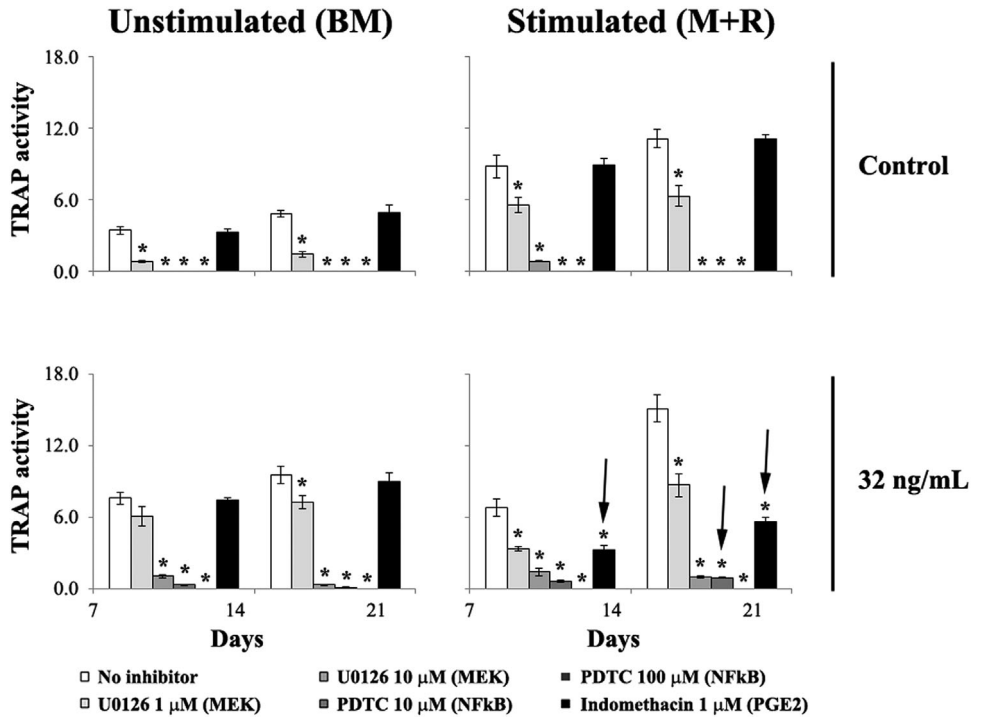

FIGURE 5 Involvement of MEK, NFkB, and PGE2 production on unstimulated (BM) and stimulated (M+R) PBMC cultures exposed to 32 ng/ml nicotine. Cell cultures were performed in the presence of the corresponding signaling pathway inhibitors, and TRAP activity was quantified. *Significantly different from cell cultures maintained in the absence of any inhibitor. Black arrows represent the main differences observed in nicotine-treated cultures, compared to the corresponding negative controls (absence of nicotine)

M+R, the production of either TNF-α or PGE2 was stimulated by nicotine (about 86% and 169%, respectively).

4 | DISCUSSION

Although it is recognized that smoking can lead to skeletal complications, affecting bone metabolism, and regeneration (Ahmed & Mohammed, 2002; Baheiraei et al., 2005; Law & Hackshaw, 1997), the effects of tobacco smoke components on bone cells and, particularly, on osteoclastic cells, are far from being elucidated. Since HA is widely used in bone regeneration therapies, due to its high similarity to bone inorganic matrix (Dimitriou et al., 2011), the present study examined the effect of nicotine on human osteoclast precursor cells cultured over a HA surface, an issue that was not previously addressed. Osteoclast precursors were cultured in the absence or in the presence of the osteoclastogenic enhancers M-CSF and RANKL, and were exposed to nicotine during 21 days. The utilization of unstimulated osteoclast precursors allows to investigate whether nicotine has the ability, by its own, to modulate osteoclastogenesis, in the absence of M-CSF and RANKL. Since osteoclast differentiation is a very complex process, potentially involving multiple different cellular mechanisms, the possibility to assess the influence of nicotine in the absence of the classic osteoclastogenic modulators M-CSF and RANKL may reveal important insights about the mechanism of action of nicotine in the modulation of osteoclastogenesis.

First, dose-response curves (1.28 ng/ml to 500 μg/ml nicotine) were established for PBMC cultured in standard cell culture plates, covering the levels reported to be present in the plasma (4–75 ng/ml) and saliva (96 ng/ml to 1.6 mg/ml) of smokers (Gritz, Baer-Weiss, Benowitz, Van Vunakis, & Jarvik, 1981; Hoffmann & Adams, 1981; James, Sayers, Drucker, & Hull, 1999; Russell, Feyerabend, & Cole, 1976; Russell, Jarvis, Iyer, & Feyerabend, 1980). Low concentrations of nicotine, 32–160 ng/ml (unstimulated cultures) and 6.4–32 ng/ml (stimulated cultures), did not affect total protein content neither apoptosis but increased TRAP activity, reaching a maximum value at 32 ng/ml. At these levels, which are in the range of those reported to be present in the plasma of smokers (Gritz et al., 1981; Russell et al., 1976, 1980), the inductive effect was higher on unstimulated PBMC, but nicotine was still able to further increase TRAP activity on the cultures supplemented with M-CSF and RANKL. This observation might suggest that nicotine may act by specific mechanisms on osteoclast precursors, which can be partially overlapped by the significant osteoclastogenic commitment elicited by the growth factors. Higher concentrations (>160 ng/ml to 500 μg/ml), which are representative of the nicotine salivary levels (Hoffmann & Adams, 1981; James et al., 1999), elicited a decrease on TRAP activity, and an increase on apoptosis (for concentrations higher than 4 μg/ml). Nicotine had a similar dose-dependent effect on TRAP activity by PBMC cultured over HA surface. Low levels (32 ng/ml) increased osteoclastogenic response, whereas levels ≥4 μg/ml elicited dose-dependent deleterious effects in all analyzed parameters, including total protein content, which suggests that nicotine at high levels may

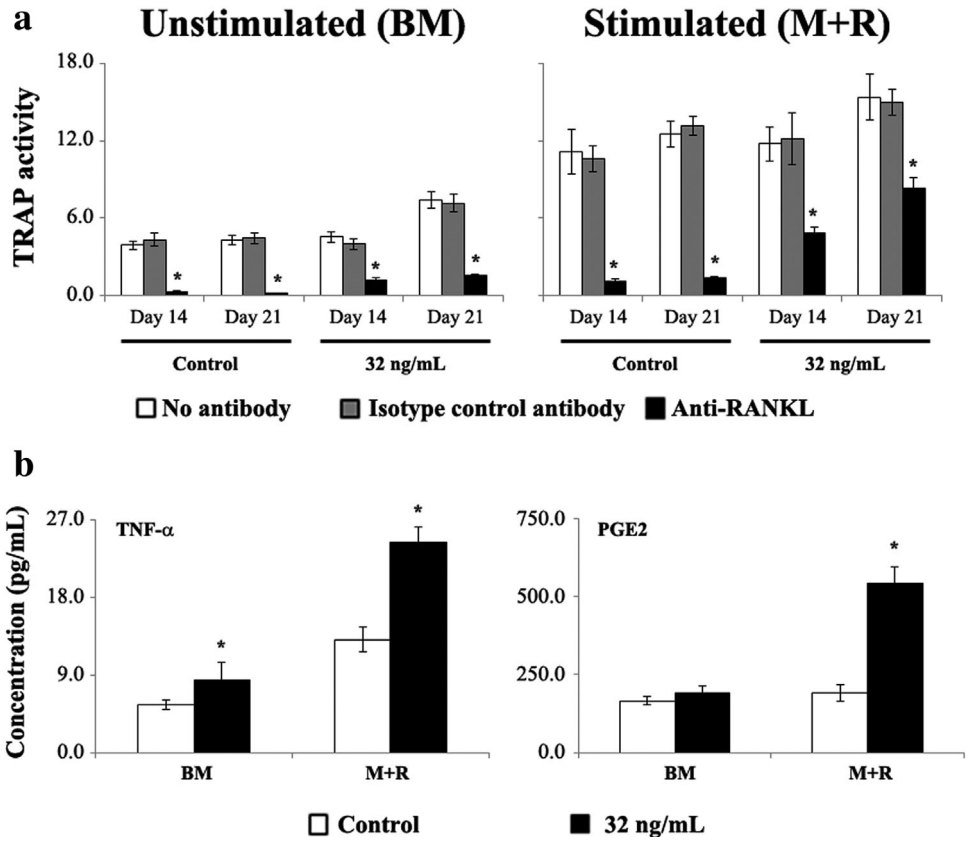

FIGURE 6 Effects of nicotine in RANKL signaling (a) and TNF-α and PGE2 production in unstimulated (BM) and stimulated (M+R) PBMC cultures performed over HA. (a) Cell cultures were treated with an isotype control antibody and with an anti-RANKL antibody and assessed for TRAP activity. (b) TNF-α and PGE2 present in culture media were quantified in the absence and presence of nicotine. *Significantly different from the control

become toxic to osteoclasts (and their precursors), promoting cell death.

As referred above, cultures performed over HA and exposed to 32 ng/ml nicotine presented increased TRAP activity. In order to understand this inductive effect, these cultures were characterized in more detail for the osteoclastogenic response and the involved mechanisms.

In unstimulated PBMC cultures, it was observed an inductive effect on TRAP activity and gene expression. However, no effects were observed in the expression of the important differentiation and activation factors c-myc and c-src (Zhao et al., 2007), neither in the functional CATK gene (Vaananen & Laitala-Leinonen, 2008). In line with this, nicotine was unable to promote an increase in the resorbing activity of these cells, as the levels of Ca^{2+} released into the culture medium remained similar to those measured in control conditions. Also, no resorption lacuna were observed in PBMC cultures maintained in BM. Putting all together, the results suggest that in the absence of M-CSF and RANKL, that means, in the absence of a strong commitment of osteoclast precursors toward osteoclastogenesis, nicotine is able to promote some degree of osteoclastic differentiation, but not the formation of fully mature bone-resorbing osteoclasts.

PBMC cultures performed in the presence of M-CSF and RANKL presented high TRAP activity, expression of osteoclastogenic genes and resorbing activity, as expected due to the role of these molecules on osteoclastic differentiation (Zhao et al., 2007). In these conditions, that is, in osteoclast precursors on-going an active osteoclastogenic differentiation, low concentrations of nicotine (32 ng/ml) caused an increase on TRAP activity and gene expression, particularly in the case of the activation factor c-src (Zhao et al., 2007) and in the gene coding for the main osteoclastic protease CATK (Vaananen & Laitala-Leinonen, 2008). The Ca^{2+} levels in the culture medium from these cultures, as well as total resorbed area, were significantly higher than those regarding control cultures, suggesting an important increase in the resorbing activity of these cells. This observation is in agreement with the SEM images of the cultures, showing evidence of increased resorption in the cultures exposed to nicotine. Thus, in opposition to what was observed in BM condition, in a context where osteoclast precursors are actively engaged in their differentiation process (M+R), nicotine appears to have the ability to potentiate osteoclastogenesis, an effect particularly evident at the level of osteoclast resorbing function.

In order to understand the mechanisms related to the pro-osteoclastogenic effect elicited by low concentrations of nicotine, the involvement of some intracellular pathways important for osteoclastogenesis was addressed. In control cultures, the NFkB pathway was essential for osteoclast development, since the presence of a specific inhibitor completely abolished TRAP synthesis. MEK pathway had also

a contribution, but PGE2 appeared not to be involved in the cell response (TRAP activity was similar in the absence or the presence of indomethacin). The most significant differences found in the cultures exposed to nicotine were a slight downregulation of the NFkB pathway (some TRAP activity in the presence of the lower concentration of the inhibitor), and an important contribution of PGE2, as the presence of indomethacin decreased significantly TRAP activity.

In order to go one step further in the characterization of the osteoclastogenic mechanisms modulated by nicotine, cell cultures were treated with anti-RANKL antibody. In control cultures, this caused a significant decrease in TRAP activity (>95%). However, in the cultures exposed to nicotine, the decrease in TRAP activity was significantly lower (~50%). This suggests that RANKL signaling may not be the sole activator of osteoclastogenesis in these experimental conditions; this is in line with the small observed downregulation elicited by nicotine in NFkB pathway (the main intracellular target of RANKL [Blair & Zaidi, 2006; Zhao et al., 2007]). Regarding this, a previous study suggested that the signaling mediated by acetylcholine receptors activated with benzopyrene may cause a decrease in RANKL-mediated osteoclastogenesis (Voronov, Heersche, Casper, Tenenbaum, & Manolson, 2005; Voronov, Li, Tenenbaum, & Manolson, 2008). Since nicotine binds to the same family of cellular receptors, the present observed cellular response regarding RANKL signaling may be related to a similar situation.

Nicotine is also known to affect inflammatory response, and there are many inflammatory molecules that play an important stimulatory role in osteoclastogenesis, such as TNF-α and PGE2 (Blair & Zaidi, 2006; Boyle et al., 2003; Zhao et al., 2007). The present study showed that nicotine caused a significant increase in the production of TNF-α (either in BM or M+R) and PGE2 (only in M+R), suggesting that nicotine may also affect osteoclastogenesis by modulating the expression of important pro-inflammatory molecules. Moreover, previous studies also showed that nicotine increased the synthesis of PGE2 by platelets and endothelial cells (Bull, Pittilo, Woolf, & Machin, 1988; Wennmalm, 1978), and by PBMC (Bernzweig, Payne, Reinhardt, Dyer, & Patil, 1998). Those results are in agreement and may also account for the observed inhibitory effect on PBMC cultures, elicited by indomethacin, in the presence of nicotine.

At the present, only three papers were published regarding the effects of nicotine on osteoclastic cells. Yuhara et al. (1999) showed that nicotine (10–250 μg/ml) reduced the formation of TRAP-positive multinucleated cells and the formation of resorption pits on slices of dentine, on osteoclast-like cells formed on co-cultures of mouse bone marrow cells and clonal stromal ST2 cells (Yuhara et al., 1999). In another study, Henemyre et al. (2003), exposed porcine mature osteoclasts to nicotine (0.03–1.50 μg/ml) and found that the number of osteoclasts increased in a linear relationship with the increase on nicotine concentration, although they did not find any correlation between osteoclast number and the amount of resorption observed (Henemyre et al., 2003). Recently, Tanaka et al. (2013) reported that nicotine caused a negative effect in osteoclast resorbing ability in RAW264.7 murine cell line treated with RANKL, for concentrations ≥16 μg/ml (Tanaka et al., 2013). Although with evident differences in the experimental protocols, these studies suggest that nicotine, in the microgram range, seems to cause negative effects on osteoclastic behavior, as it was observed in the present work with human cells. The variability in the reported cell response might also be related to the different cell types used, that is, rat, porcine and murine cells, stressing the relevance of using human primary osteoclastic cells as a more representative model to address this issue. Regarding the intracellular mechanisms modulated by nicotine, no previously published works addressed this issue.

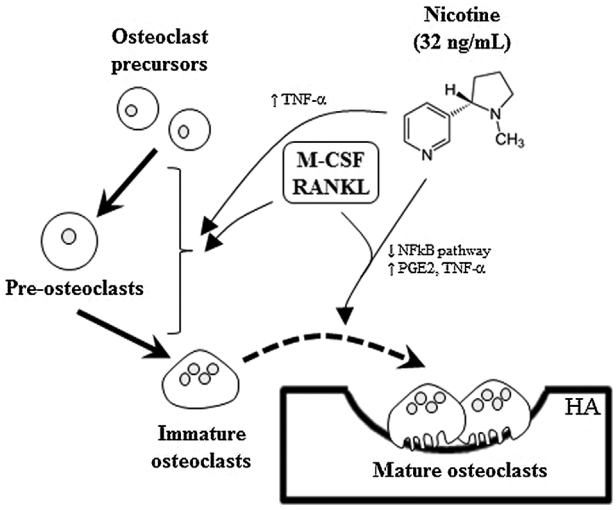

FIGURE 7 Proposed mechanism of action of nicotine in human osteoclastogenesis over hydroxyapatite

In conclusion, this study demonstrate that, in PBMC cultures, nicotine has the ability to act directly on human osteoclastic precursors, inducing (low concentrations) or inhibiting (high concentrations) osteoclast differentiation. The inductive effect appears to be dependent on the stage of osteoclastic differentiation (Figure 7). On unstimulated precursors, nicotine increased TRAP activity and the expression of several osteoclast-related genes, but did not significantly affect the resorbing activity, which suggests that in this condition nicotine only stimulates the first steps of osteoclastogenesis, but it is not able to complete the process. By contrast, on osteoclastic precursors engaged on a differentiation process, which ends up in mature osteoclasts, nicotine greatly increased not only their differentiation, but also their resorption activity. The modulation of RANKL signaling and the expression of inflammatory molecules like TNFα and PGE2 appeared to be involved in the inductive effect of nicotine. Taken together, this is the first report with data on the effects of nicotine on human osteoclast precursors cultured over HA. Results showed that nicotine has dose-dependent effects on osteoclastic behavior, with together with the reported effects over osteoblastic cells, might contribute to disrupt the bone metabolic activities and, consequently, normal bone remodeling at the bone/biomaterial interface.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

Ahmed, Y. G., & Mohammed, M. B. (2002). Effect of cigarette smoking on human PDL fibroblasts attachment to periodontally involved root surfaces in vitro. *Journal of Clinical Periodontology*, *29*, 763–770.

Baheiraei, A., Pocock, N., Eisman, J., Nguyen, N., & Nguyen, T. (2005). Bone mineral density, body mass index and cigarette smoking among Iranian women: Implications for prevention. *BMC Musculoskeletal Disorders*, *6*, 34.

Balatsouka, D., Gotfredsen, K., Lindh, C. H., & Berglundh, T. (2005). The impact of nicotine on bone healing and osseointegration. *Clinical Oral Implants Research*, *16*, 268–276.

Bernzweig, E., Payne, J. B., Reinhardt, R. A., Dyer, J. K., & Patil, K. D. (1998). Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *Journal of Clinical Periodontology*, *25*, 246–252.

Blair, H. C., & Zaidi, M. (2006). Osteoclastic differentiation and function regulated by old and new pathways. *Reviews in Endocrine and Metabolic Disorders*, *7*, 23–32.

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature*, *423*, 337–342.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Bull, H. A., Pittilo, R. M., Woolf, N., & Machin, S. J. (1988). The effect of nicotine on human endothelial cell release of prostaglandins and ultrastructure. *British Journal of Experimental Pathology*, *69*, 413–421.

Costa-Rodrigues, J., Teixeira, C., & Fernandes, M. (2011). Paracrine-mediated osteoclastogenesis by the osteosarcoma MG63 cell line: Is RANKL/RANK signalling really important? *Clinical and Experimental Metastasis*, *28*, 505–514.

Costa-Rodrigues, J., Fernandes, A., Lopes, M. A., & Fernandes, M. H. (2012). Hydroxyapatite surface roughness: Complex modulation of the osteoclastogenesis of human precursor cells. *Acta Biomaterialia*, *8*, 1137–1145.

Datta, H. K., Ng, W. F., Walker, J. A., Tuck, S. P., & Varanasi, S. S. (2008). The cell biology of bone metabolism. *Journal of Clinical Pathology*, *61*, 577–587.

Dimitriou, R., Jones, E., McGonagle, D., & Giannoudis, P. V. (2011). Bone regeneration: Current concepts and future directions. *BMC Medicine*, *9*, 66.

Gritz, E. R., Baer-Weiss, V., Benowitz, N. L., Van Vunakis, H., & Jarvik, M. E. (1981). Plasma nicotine and cotinine concentrations in habitual smokeless tobacco users. *Clinical Pharmacology & Therapeutics*, *30*, 201–209.

Hall, T. J., Schaeublin, M., Jeker, H., Fuller, K., & Chambers, T. J. (1995). The role of reactive oxygen intermediates in osteoclastic bone resorption. *Biochemical and Biophysical Research Communications*, *207*, 280–287.

Henemyre, C. L., Scales, D. K., Hokett, S. D., Cuenin, M. F., Peacock, M. E., Parker, M. H., … Chuang, A. H. (2003). Nicotine stimulates osteoclast resorption in a porcine marrow cell model. *Journal of Periodontology*, *74*, 1440–1446.

Hoffmann, D., & Adams, J. D. (1981). Carcinogenic tobacco-specific N-Nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Research*, *41*, 4305–4308.

Hotokezaka, H., Sakai, E., Kanaoka, K., Saito, K., K-i, M., Kitaura, H., … Nakayama, K. (2002). U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. *The Journal of Biological Chemistry*, *277*, 47366–47372.

James, J. A., Sayers, N. M., Drucker, D. B., & Hull, P. S. (1999). Effects of tobacco products on the attachment and growth of periodontal ligament fibroblasts. *Journal of Periodontology*, *70*, 518–525.

Jeffrey, O. H., John, M. S., Kun, H., Peiman, S., & Dave, B. (1999). Impact of nicotine on bone healing. *Journal of Biomedical Materials Research*, *45*, 294–301.

Kawashima, M., Fujikawa, Y., Itonaga, I., Takita, C., & Tsumura, H. (2009). The effect of selective cyclooxygenase-2 inhibitor on human osteoclast precursors to influence osteoclastogenesis in vitro. *Modern Rheumatology*, *19*, 192–198.

Kellinsalmi, M., Parikka, V., Risteli, J., Hentunen, T., Leskelä, H.-V., Lehtonen, S., … Lehenkari, P. (2007). Inhibition of cyclooxygenase-2 down-regulates osteoclast and osteoblast differentiation and favours adipocyte formation in vitro. *European Journal of Pharmacology*, *572*, 102–110.

Kim, H. J., Lee, Y., Chang, E.-J., Kim, H.-M., Hong, S.-P., Lee, Z. H., … Kim, H.-H. (2007). Suppression of osteoclastogenesis by N,N-Dimethyl-D-erythro-sphingosine: A sphingosine kinase inhibition-Independent action. *Molecular Pharmacology*, *72*, 418–428.

Law, M. R., & Hackshaw, A. K. (1997). A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: Recognition of a major effect. *BMJ*, *315*, 841–846.

Pereira, M. L., Carvalho, J. C., Peres, F., & Fernandes, M. H. (2009). Effect of nicotine in matrix mineralization by human bone marrow and Saos-2 cells cultured on the surface of plasma-sprayed titanium implants. *Journal of Biomedocal Materials Resarch Part A*, *88A*, 84–93.

Pereira, M. L., Carvalho, J. C., Peres, F., Gutierres, M., & Fernandes, M. H. (2008). Behaviour of human osteoblastic cells cultured on plasma-sprayed titanium implants in the presence of nicotine. *Clinical Oral Implants Research*, *19*, 582–589.

Rumpel, E., Wolf, E., Kauschke, E., Bienengräber, V., Bayerlein, T., Gedrange, T., & Proff, P. (2006). The biodegradation of hydroxyapatite bone graft substitutes in vivo. *Folia Morphologica*, *65*, 43–48.

Russell, M. A., Feyerabend, C., & Cole, P. V. (1976). Plasma nicotine levels after cigarette smoking and chewing nicotine gum. *British Medical Journal*, *1*, 1043–1046.

Russell, M. A., Jarvis, M., Iyer, R., & Feyerabend, C. (1980). Relation of nicotine yield of cigarettes to blood nicotine concentrations in smokers. *British Medical Journal*, *280*, 972–976.

Seiichi, Y., Joel, A. B., Winston, P. K., German, O. G., Hans-Peter, W., & Cortino, S. (2010). Effects of nicotine on gene expression and osseointegration in rats. *Clinical Oral Implants Research*, *21*, 1353–1359.

Tanaka, H., Tanabe, N., Kawato, T., Nakai, K., Kariya, T., Matsumoto, S., … Maeno, M. (2013). Nicotine affects bone resorption and suppresses the expression of cathepsin K, MMP-9 and vacuolar-type H(+)-ATPase d2 and actin organization in osteoclasts. *PLoS ONE*, *8*, e59402.

Vaananen, H. K., & Laitala-Leinonen, T. (2008). Osteoclast lineage and function. *Archives of Biochemistry and Biophysics*, *473*, 132–138.

Voronov, I., Li, K., Tenenbaum, H. C., & Manolson, M. F. (2008). Benzo[a]pyrene inhibits osteoclastogenesis by affecting

RANKL-induced activation of NF-kappaB. *Biochemical Pharmacology*, *75*, 2034–2044.

Voronov, I., Heersche, J. N., Casper, R. F., Tenenbaum, H. C., & Manolson, M. F. (2005). Inhibition of osteoclast differentiation by polycyclic aryl hydrocarbons is dependent on cell density and RANKL concentration. *Biochemical Pharmacology*, *70*, 300–307.

Wennmalm, A. (1978). Effects of nicotine on cardiac prostaglandin and platelet thromboxane synthesis. *British Journal of Pharmacology*, *64*, 559–563.

Yang, Q., McHugh, K. P., Patntirapong, S., Gu, X., Wunderlich, L., & Hauschka, P. V. (2008). VEGF enhancement of osteoclast survival and bone resorption involves VEGF receptor-2 signaling and [beta]3-integrin. *Matrix Biology*, *27*, 589–599.

Yuhara, S., Kasagi, S., Inoue, A., Otsuka, E., Hirose, S., & Hagiwara, H. (1999). Effects of nicotine on cultured cells suggest that it can influence the formation and resorption of bone. *European Journal of Pharmacology*, *383*, 387–393.

Zhao, Q., Shao, J., Chen, W., & Li, Y. P. (2007). Osteoclast differentiation and gene regulation. *Frontiers in Bioscience: A Journal and Virtual Library*, *12*, 2519–2529.