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Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells

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ABSTRACT

Semi-synthetic tetracyclines (TCs) have been reported to reduce pathological bone resorption through several mechanisms, although their effect over bone physiological metabolism is not yet fully understood. The present study aims at evaluate the behaviour of osteoblastic-induced human bone marrow cells regarding proliferation and functional activity, in the presence of representative therapeutic concentrations of doxycycline and minocycline. First passage human osteoblastic bone marrow cells were cultured for 35 days in conditions known to favor osteoblastic differentiation. Doxycycline (1–25 μg/ml) or minocycline (1–50 μg/ml) were added continuously, with the culture medium, twice a week with every medium change. Cultures were characterised at several time points for cell proliferation and function.

Present data showed that 1 μg/ml of both tetracyclines, level representative of that attained in plasma and crevicular fluid with the standard therapeutic dosage, increased significantly the proliferation of human bone marrow osteoblastic cells without altering their specific phenotype and functional activity. Long-term exposure to these TCs induced a significant increase in the number of active osteoblastic cells that yielded a proportional amount of a normal mineralised matrix, suggesting a potential application in therapeutic approaches aiming to increase bone formation. The presence of higher levels of these agents led to a dose-dependent deleterious effect over cell culture, delaying cell proliferation and differentiation.

1. Introduction

An emerging concept on periodontal therapy is the need to block the host response and the bacterial action since both are responsible for tissue damage.[1] Bacterial biofilms are known to be associated to the initiation of the inflammatory response as well as periodontal destruction, but the major component of periodontal disease is related to the activation of the host immuno-inflammatory response.[2] For instance, most of the soft- and hard-tissue damage (clinically characterised by pocketing, loss of attachment and alveolar bone destruction) occurs when synthesis of endothelial and intercellular adhesion molecules is over-expressed in association with several immune and inflammatory mediators. Inflammatory cells as well as fibroblasts, osteoblasts, epithelial and other structural cells are responsible for the release of several mediators such as prostaglandins, cytokines, eicosanoids, matrix metalloproteinases

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(MMPs), among others, that are know to mediate the immuno-inflammatory response.[3]

Evidence converge to the realisation that an imbalance between pro-inflammatory and anti-inflammatory cytokines is linked with the pathogenesis of several diseases associated with bone loss.[4,5] In such diseases, many therapeutic mechanisms have been proposed to block bone resorption and promote bone formation, including gene therapy, administration of bioactive molecules like BMPs, growth factors and MMPs inhibitors.[6,7] Regarding clinical application of MMPs inhibitors, several properties of tetracyclines (TCs) have been reported to contribute to their therapeutic effectiveness in this situation. These include cation-quelation activity, with consequent avidity for mineralised tissues and MMPs inactivation, and long-term clinical safety, all independent from antimicrobial activity.[8,9] Recently, subantimicrobial doses of doxycycline (SDD) have been approved as a host response modifier in the treatment of periodontal disease, and positive effects were observed in clinical trials in patients with chronic periodontitis receiving this therapeutic approach.[10–12]

Several reports tend to indicate a bone forming activity by TCs. Most investigations were based on pathological induced bone diseases in animal models, and positive effects were obtained on rheumatoid arthritis,[13] osteoporosis condition,[14] diabetes induced osteopenia,[15–18] cartilage degradation[19–21] and bone loss induced by estrogens deficiency.[22] A recent study evaluated the effect of tetracycline upon morphologic characteristics of bone undergoing normal remodeling in squirrel monkeys and also found increased osteoblastic activity and osteoid formation in alveolar bone.[23] Several mechanisms have been proposed to explain the benefic effect including enhancing of bone formation, decreasing of connective tissue breakdown and diminishing of bone resorption;[16–24] the most widely investigated is related to the ability of these agents to inhibit the activity of MMPs. The MMPs family is responsible for most of the degradation process of the constituent macromolecules located in the extracellular matrix of several tissues, including periodontium.[24] They are active at physiological pH and their main function is to promote protein breakdown in cell membrane and in the extracelullar matrix, which is known to play an essential role in the proliferation and differentiation of cellular populations of the conjunctive tissues,[25–29] including osteoblastic cells.[25,26,29,30]

To the best of our knowledge, the dose-dependent response of human osteoblastic cells to TCs has not been addressed previously. In this way, to further comprehend the mechanisms associated with the eventual anabolic effect of TCs over bone metabolism, this work evaluated the proliferation and functional activity of osteoblastic-induced human bone marrow cells in the presence of selected concentrations of doxycycline and minocycline, representative of those found in plasma and crevicular fluid after oral administration.

2. Materials and methods

2.1. Cell culture

Bone marrow was obtained from patients undergoing orthopaedic surgery procedures. Informed consent to use this biological material, that would be otherwise discarded, was obtained. Bone marrow was cultured in α-minimal essential medium (α-MEM) containing 10% foetal bovine serum, 50 μg/ml gentamicin, 2.5 μg/ml fungizone and 50 μg/ml ascorbic acid. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C, and the medium was changed twice a week. Primary cultures were maintained for 10/15 days till near confluence when adherent cells were enzymatically released with 0.04% trypsin and 0.025% collagenase. The resultant cell suspension was cultured (10^4 cell/cm^2) at the same experimental conditions, but the culture medium was further supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone. Cell cultures were established for 35 days in absence (control) or presence of doxycycline (1–25 μg/ml) or minocycline (1–50 μg/ml). TCs were renewed in the culture at each medium change, twice a week. Cell cultures were routinely monitorised by phase contrast optic microscopy and characterised at days 3, 7, 14, 21, 28 and 35 for cell proliferation and function.

2.2. Culture characterisation

2.2.1. Cell viability/proliferation and total protein content

Proliferation studies included MTT assay and total protein content. MTT assay was based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. In the last 4 h of each test time, cells were incubated with 0.5 mg/ml of MTT in the conditions referred above. The medium was then decanted and the stained product dissolved with dimethylsulphoxide before absorbance determination at 600 nm.

Total protein content was determined by Lowry method, after treatment of the cell layer with 0.1 M NaOH for 1 h. Bovine serum albumin was used as a standard, and absorbance evaluated at 750 nm.

2.2.2. Alkaline phosphatase (ALP) activity

ALP was determined in cell lysates (0.1% triton) by the hydrolysis (30 min at 37 °C) of *p*-nitrophenyl phosphate in an alkaline buffer solution (pH 10.3), followed by colorimetric determination of *p*-nitrophenol at 405 nm. Results were expressed in nanomoles of *p*-nitrophenol produced per minute per μg of protein (nmol/min/μg protein).

2.2.3. Histochemical staining for calcium and phosphate deposition

Fixed cultures (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) were stained for the presence of calcium and phosphate deposits in the extracellular matrix.

For calcium staining, the fixed cultures were covered with a 1% S alizarin sodium solution (0.028% in NH_4OH), pH 6.4, for 2 min and then rinsed with distilled water and acid ethanol (ethanol, 0.01% HCL). Calcium deposits stained red.

Phosphate deposits were assessed by the von Kossa assay. Fixed cultures were treated with a 1.0% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

2.2.4. *Scanning electron microscopy (SEM)*

Fixed cultures were dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JeoL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDX) microanalysis capability (Voyager XRMA System, Noran Instruments).

2.2.5. *Ionised calcium (Ca_i) and phosphorus (P_i) in the culture medium*

Culture media from control and TCs-treated cultures were collected every 2–3 days (and cultures reefed with fresh medium) during the 35 days culture time and analysed for Ca_i and P_i concentration. Ca_i and P_i were dosed using Sigma Diagnostics Kits, respectively procedures number 587 and 670. Results were expressed in mmol per litre of medium (mmol/l).

2.3. *Statistical analyses*

Results presented in this study are from three separate experiments using cell cultures from three different donors (both sexes and aged between 20 and 50 years old). For each experiment and essay, eight replicas were accomplished. Groups of data were evaluated using a one-way analysis of variance (ANOVA) and no significantly differences in the pattern of the cell behaviour were found. Statistical differences found between control and TC-treated cultures were determined by Bonferroni's method with significance set up to $p \leq 0.05$.

3. Results

Human bone marrow cells (first subculture) were characterised for proliferation and differentiation events during 35 days. Cultures were grown in an osteoblastic-inducing medium in the presence of a concentration range of doxycycline (1–25 μg/ml) or minocycline (1–50 μg/ml).

3.1. *Cell viability/proliferation*

Results regarding cell proliferation were measured by the MTT assay in the various experimental conditions and are reported in Fig. 1A.

Cultures grown in control conditions showed a gradual increase in the cell proliferation till day 21, followed by a decrease in the last 2 weeks. Doxycycline and minocycline caused an initial dose-dependent inhibitory effect (lower MTT reduction at day 3), followed by an increase in the cell proliferation. This initial negative effect ranged from 2 to 3 days in the presence of 1 μg/ml, for both TCs, to progressively longer periods at higher levels. The continuous exposure to doxycycline (1 and 5 μg/ml) and minocycline (1–10 μg/ml) caused an evident induction of cell proliferation from the first week onwards. At day 21, the MTT reduction values were around two-fold higher in the presence of 1 and 5 μg/ml, as compared to control cultures. However, cultures treated with levels higher than 1 μg/ml presented a delayed maturation, since maximal

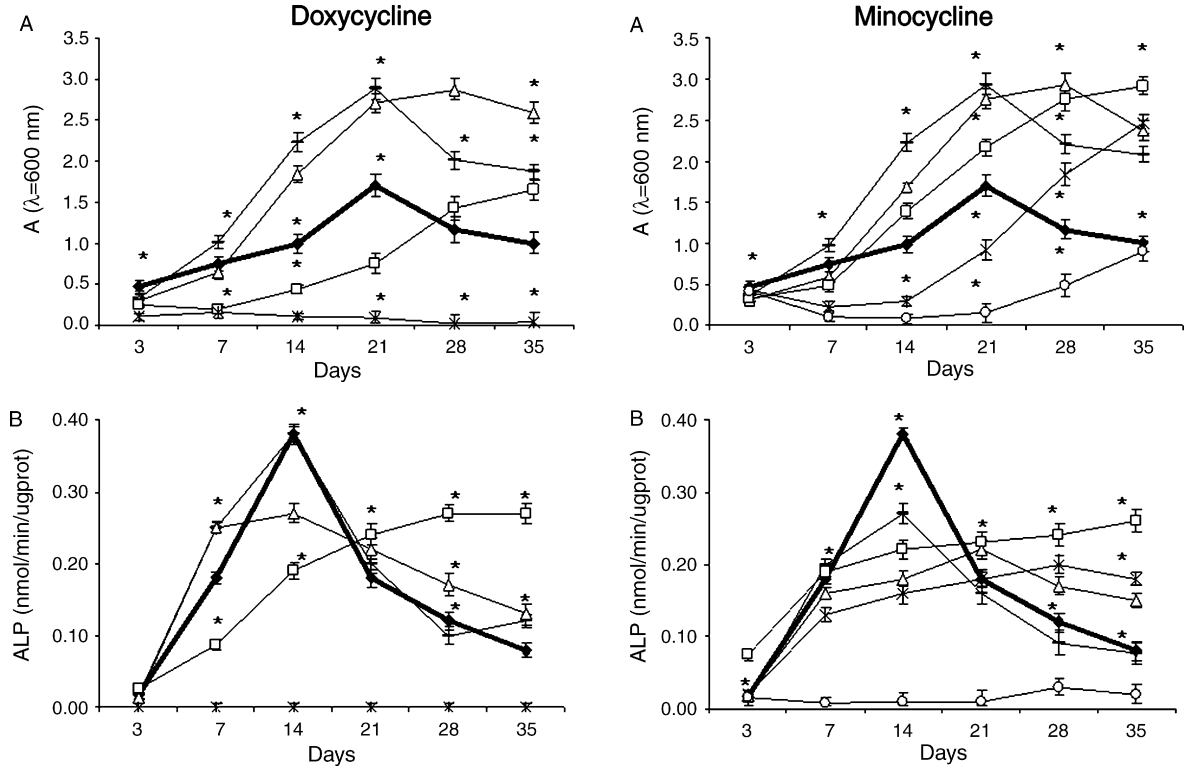

Fig. 1 – Cell viability/proliferation (A) and alkaline phosphatase activity (B) of human osteoblastic bone marrow cells cultured for 35 days and exposed continuously to doxycycline or minocycline. Control cultures (◆, embossed line). Cultures treated with tetracyclines: 1 μg/ml (–), 5 μg/ml (△), 10 μg/ml (□), 25 μg/ml (*) and 50 μg/ml (○). *Significantly different from control cultures ($p \leq 0.05$).

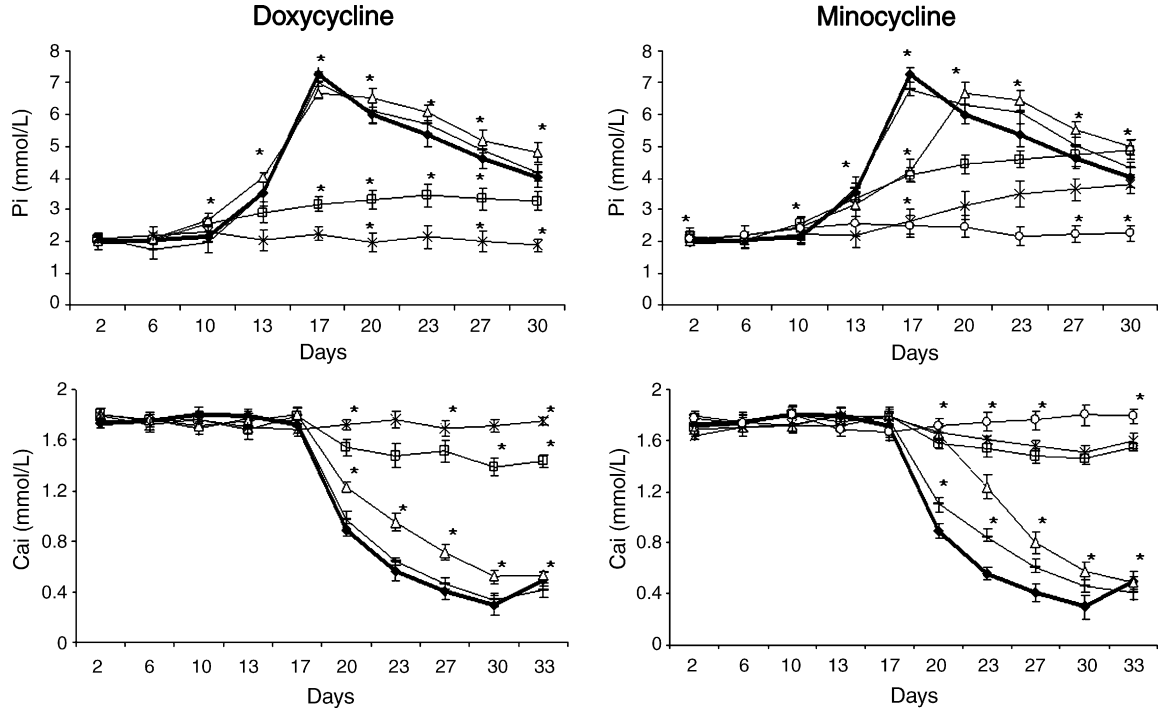

Fig. 2 – Levels of ionised phosphorus (P_i) and calcium (Ca_i) in the culture medium from human osteoblastic bone marrow cell cultures, collected at each medium change (twice a week) during the 35-day incubation time. Therefore, levels measured were not cumulative, reflecting changes occurring in intervals of 2–3 days throughout the culture period. Control cultures (◆, embossed line). Cultures treated with tetracyclines: 1 μg/ml (–), 5 μg/ml (△), 10 μg/ml (□), 25 μg/ml (*) and 50 μg/ml (○). *Significantly different from control cultures ($p \leq 0.05$).

proliferation was achieved later. Doxycycline, at 10 μg/ml, caused a significant inhibitory effect in cell growth with a recovery at late incubation time, and treatment with 25 μg/ml resulted in an almost absence of cell growth. At this high concentration range, minocycline presented lower long-term cytotoxic potential, with cell growth being observed in the presence of 25 and 50 μg/ml, although after a lag phase of 2 and 3 weeks, respectively.

Results regarding total protein content reflected similar information as that obtained from the MTT assay (not shown).

3.2. *Alkaline phosphatase activity*

3.2.1. *Results concerning ALP activity are shown in Fig. 1B*
Control cultures presented ALP levels that increased with incubation time till day 14, decreasing significantly after that. Doxycycline, at 1 μg/ml, did not affect cell behaviour, but treatment with 5 μg/ml caused a slight decrease in maximal levels, although with a similar pattern of expression. The addition of 10 μg/ml resulted in a significant initial decrease, but with the cells progressively recovering the ability to synthesise this enzyme. In the presence of 25 μg/ml, values were very low, reflecting the almost absence of cell growth in this situation. Treatment with minocycline resulted in a higher dose-dependent inhibitory effect in ALP activity, and decreased maximal levels were observed even in the presence of 1 μg/ml. Negligible levels were measured in cultures exposed to 50 μg/ml.

3.3. *Matrix mineralisation*

The presence of calcium phosphate deposits in the extracellular matrix was inferred from the pattern of variation of Ca_i and P_i in the culture medium during the incubation time (Fig. 2) and visualised by histochemical staining (Table 1, Fig. 3) and SEM observation (Fig. 4).

Table 1 – Staining intensity resulting from histochemical assessment of calcium and phosphate deposits (Alizarin red and von Kossa assays) in human osteoblastic bone marrow cell cultures exposed continuously to doxycycline or minocycline, days 14–35

	Days			
	14	21	28	35
Control	–	+	+++	+++
Doxycydine				
1 μg/ml	–	++	+++	+++
5 μg/ml	–	++	++	++
10 μg/ml	–	–	–	+
Minocycline				
1 μg/ml	–	++	+++	+++
5 μg/ml	–	±	+	+
10 μg/ml	–	–	–	+

Intensity of staining was graded as follows: (–) negative staining; (±) fair/absent staining; (+) definite staining but of low intensity; (++) moderate staining; (+++) intense staining.

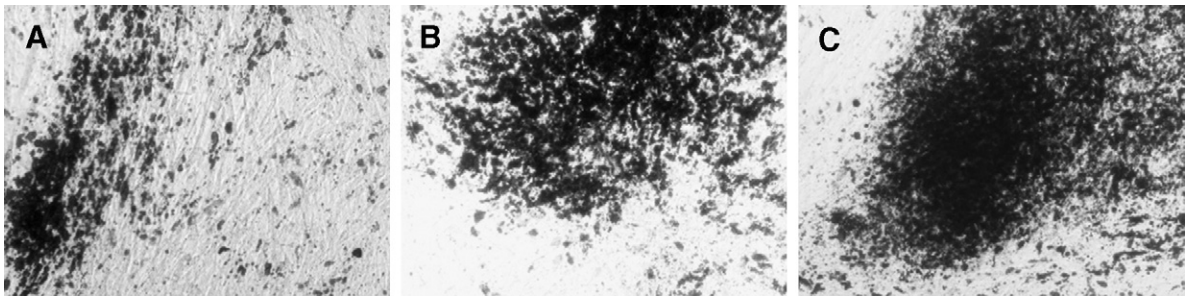

Fig. 3 – Histochemical assessment of mineral deposition in human osteoblastic bone marrow cell cultures. Representative von Kossa staining at days 21 (A and B) and 35 (C). (A) Control culture; (B and C) cultures treated with doxycycline 1 μg/ml. Magnification 100×.

3.3.1. *P_i and Ca_i levels in the culture medium*

P_i and Ca_i in the culture medium (approximately 2 and 1.8 mmol/l, respectively) originated from calcium and phosphate compounds present in α-MEM and in foetal bovine serum. In addition, β-glycerophosphate added to the culture medium (10 mmol/l) provided a source of P_i, after being hydrolysed by the increasing levels of ALP present in the cultures. Consumption of P_i and Ca_i from the culture medium reflects the mineralisation process, i.e. the formation of calcium phosphate deposits in the extracellular matrix. In

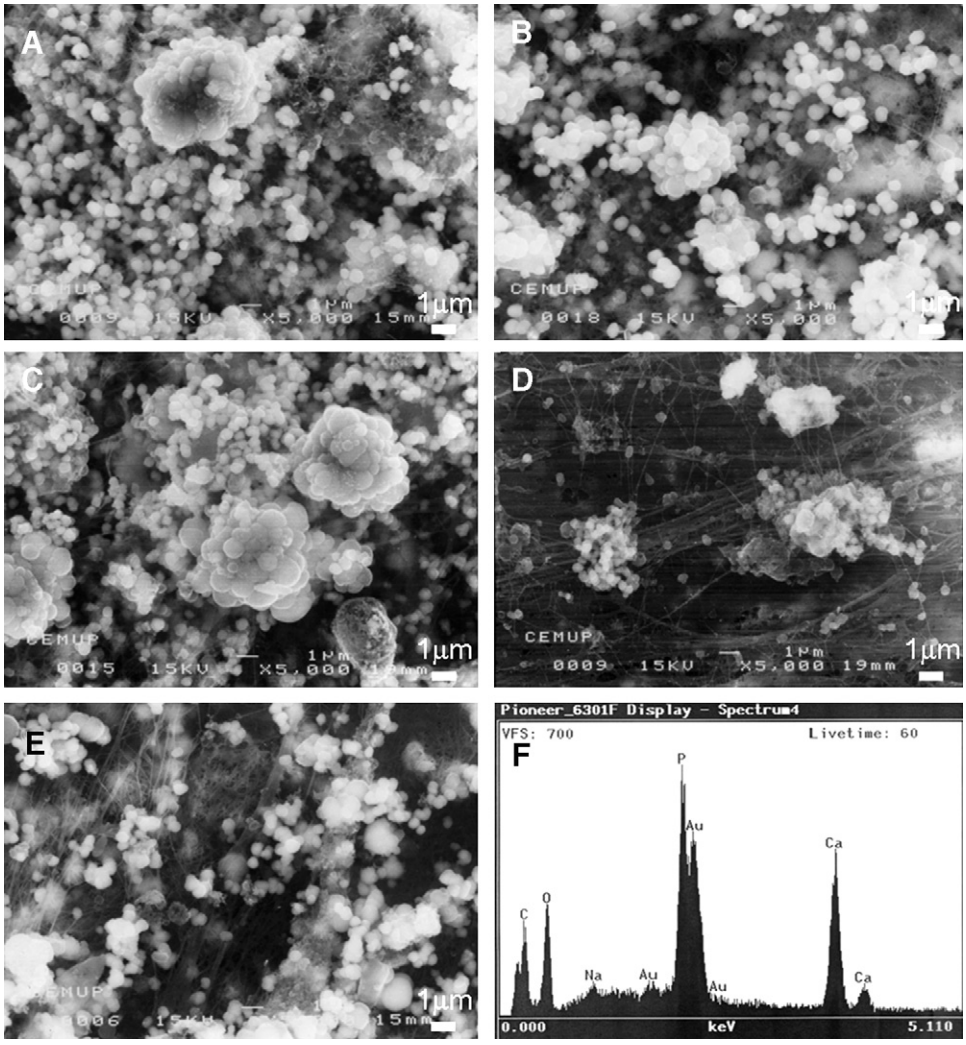

Fig. 4 – SEM appearance of human osteoblastic bone marrow cell cultures exposed continuously to doxycycline or minocycline, at day 28. (A) 1 μg/ml doxycycline; (B) 5 μg/ml doxycycline; (C) 1 μg/ml minocycline; (D) 5 μg/ml minocycline; (E) control conditions; (F) representative X-ray spectrum of the mineralised deposits.

the present work, levels of P_i and Ca_i measured were not cumulative, as culture medium was totally replaced at each medium change. In this way, values reflect changes occurring in intervals of 2–3 days throughout the culture period, providing quantitative information regarding the ongoing mineral deposition in control and TCs-treated cultures.

Medium collected from control cultures presented similar P_i levels for the first 10 days; after that, values increased until day 17 (attaining 7.5 mmol/l), decreasing afterwards. Ca_i levels were constant until around day 17 and decreased significantly after that (0.3 mmol/l, at day 30). Exposure to doxycycline, 1 and 5 μg/ml, and minocycline, 1 μg/ml, resulted in behaviour similar to control, suggesting an identical pattern of calcium phosphate deposition. In the presence of 5 μg/ml minocycline, mineral deposition began few days later and occurred, apparently, at a lower rate; this observation is suggested by the levels of P_i (maximal values were slightly lower and attained later, around day 20) and Ca_i (slower rate of decrease, with the onset around day 20). Cultures treated with doxycycline, 10 μg/ml, or minocycline, 10 and 25 μg/ml, presented an evident impairment in the production of P_i, reflecting the lower production of ALP in these conditions. Also, only a slight consumption of Ca_i and P_i from the medium was observed, suggesting a late and faint mineral deposition. Ca_i and P_i levels remained constant in the medium collected from the cultures treated with 25 μg/ml doxycycline or 50 μg/ml minocycline. Results are shown in Fig. 2.

3.3.2. *Histochemical staining of calcium deposits. SEM observation*

In control cultures, matrix mineralisation occurred during the third week. Cultures presented a positive staining for calcium and phosphate deposits from day 21 onwards and the mineral deposition increased with the incubation time. In addition, staining was especially evident in areas of high cell density, with the appearance of nodule-like structures. SEM observation provided similar information. Cultures presented a continuous cell layer with abundant mineral globular deposits closely associated with the fibrous matrix showing the presence of Ca and P peaks on X-ray microanalysis.

Cultures treated with the lower concentrations of doxycycline (1 and 5 μg/ml) and minocycline (1 μg/ml) presented similar behaviour to that of control cultures, but a higher abundance of mineral deposition. Comparatively, lower matrix mineralisation was observed in the cultures exposed to 5 μg/ml minocycline. In the presence of doxycycline and minocycline, at 10 μg/ml, mineral deposition was observed only at day 35.

Results are summarised in Table 1 regarding the staining intensity resulting from histochemical assessment of calcium and phosphate deposits. Figs. 3 and 4 show the appearance of the cultures, respectively light micrographs of stained cultures and scanning electron micrographs. The areas shown are representative of the corresponding cell layer at the experimental conditions selected.

4. Discussion

Human bone marrow cells were cultured in α-MEM with foetal bovine serum, ascorbic acid, β-glycerophosphate and dexamethasone, conditions known to favor the development of osteoblastic phenotype.[2] Based on this premise, characterisation of the cell behaviour of cultures treated continuously with a selected concentration range of semi-synthetic tetracyclines – doxycycline (1–25 μg/ml) and minocycline (1–50 μg/ml) – was assayed.

Bone marrow cells grown in control conditions presented a phase of active proliferation during the first 3 weeks (Fig. 1A) and synthesis of high levels of ALP (Fig. 1B). Activity of this enzyme increased especially during the second week, suggesting a significant differentiation of the cells in this stage of the culture. ALP appears to play an essential role in the mineralisation process providing high levels of phosphate ions to the onset of mineral deposition, being subsequently down-regulated.[29,31] In the present work, hydrolysis of β-glycerophosphate by ALP led to a significant increase in the levels of P_i in the culture medium until day 17, with maximal values being around 8 mmol/l (Fig. 2), suggesting that most of this compound was converted to inorganic phosphate. According to the pattern of Ca_i and P_i in the medium (Fig. 2), mineral deposition began to occur around day 17 and this was confirmed by the positive staining on Alizarin red and von Kossa assays (Table 1, Fig. 3) and the presence of calcium phosphate deposits intimately associated with the cell layer on SEM observation, from day 21 onwards (Fig. 4). In the last 2 weeks, the osteoblastic cells became progressively embedded by the mineralising matrix and unable to proliferate, as apparent by the decreased MTT reduction values measured at days 28 and 35 (Fig. 1A). This behaviour is similar to previous results[32] and in line with the established model for the *in vitro* development of the osteoblastic phenotype.[26] Human bone marrow cells in culture presented a continually changing differentiation status, namely a proliferative and poor differentiated cell population, at early time points, and a less proliferative and more differentiated cell population, at later time points, ending with the formation of a cell-mediated mineralised matrix, the event that represents the complete expression of the osteoblastic phenotype.[26]

The present data suggest that low doxycycline and minocycline concentrations are able to stimulate the proliferation of osteoblastic-induced bone marrow cells. After an initial inhibitory effect observed during the first 2–3 days, treatment with 1 μg/ml caused an increase in the cell growth, maintenance of ALP activity and higher abundance of mineral deposition. Exposure to 5 μg/ml doxycycline resulted in a similar behaviour. Comparatively, minocycline, at this concentration, caused a slight impairment in osteoblastic function. Higher TCs levels delayed cell proliferation and function. The selected concentration range is of clinical significance as pharmacokinetic studies showed that after oral uptake of 200 mg of doxycycline or minocycline (usual therapeutic dosage), the plasma concentration reached 3 μg/ml at 2 h and was maintained above 1 μg/ml for 8–12 h.[33] Similar and higher levels were found in GCF.[33,34] On the other hand, therapeutic dosage of SDD (20 mg twice daily) resulted in peak serum concentrations around 0.8 μg/ml and steady-state concentrations around 0.48 μg/ml.[35]

The effects of TCs over osteoblastic cells were addressed in few previous studies, although in different cell systems and conditions. Williams et al. reported that minocycline, at levels

up to 3 μg/ml, might increase the efficiency of rat's primary bone marrow stromal cells regarding colony formation capacity.[36] Schartz et al. reported induction of early differentiation events and cell proliferation improvement with diminished later differentiation events (like osteocalcin production) in osteoblast-like MG63 cells cultured in tetracycline pre-treated dentin.[37] In contrast, Homes et al. reported that doxycycline (up to 5 μg/ml) addition to the culture of mouse osteoblast-precursors did not result in any anabolic effect in cultures established for 12 days and exposed between 0 and 5, 5 and 9 or 9 and 12 days.[38] Differences among studies might be related with the cell system used (human/animal cells, stage of cell differentiation), the pharmacological agent analysed and the type of exposure (continuous/intermittent). This parameter appears to be relevant, as the present results showed that, for a given dose, the effect on cell proliferation is time-dependent, i.e. an initial inhibitory effect followed by an induction on long-term treatment. This probably explains, at least in part, the absence of effect reported by Homes et al. that tested short and intermittent doxycycline exposures.

The results of the present *in vitro* study are in line with those reported by several authors investigating bone metabolism and pathology in animal models. The mouse model was used by Golub et al. to simulate diabetic-induced osteopenia in which it was verified that the pathological situation (characterised by decreased bone formation and not augmented bone resorption) was significantly ameliorated with tetracycline uptake.[15] Later, Sasaki et al. suggested that minocycline and CMTs would be able to improve osteoblastic structure and function in diabetes-induced osteopenia in rats.[16] Li et al. reported increased bone formation and mass levels on ovariectomised rats by low dose tetracycline administration. The two concentrations tested reflected 0.24–0.72% of clinical antibiotic regular dose and while the lower dose increased osteoblastic recruitment, the higher increased osteoblastic activity.[39] Recently, tetracycline administration to squirrel monkeys, in a model of normal bone metabolism, increased the deposition of osteoid in the alveolar process by increasing the number of active osteoblasts.[23]

The present data tend to indicate that low concentrations of doxycycline and minocycline – 1 μg/ml – have proanabolic effects over osteoblastic phenotypic cells, regarding proliferation. These TCs, that apparently need long treatment periods (several days) to cause the stimulating effects, may act as promoters of less differentiated stages inducing cell growth, without altering functional activity. This observation is suggested by the significantly increased cell proliferation in the TCs-treated cultures, but similar ALP activity; in this way, the higher abundance of mineral deposition is likely related to the increased number of viable and normal functioning cells in culture. However, in the present work, TCs were present throughout the culture time and, therefore, the effect of these agents was assessed in conditions of a continually changing differentiation status of the culture, making data interpreting difficult. Drug exposure at early and late time points would provide information regarding the responsiveness of osteoblastic cells at different stages of cell differentiation. The mechanism by which these TCs exert their favourable effects over osteoblasts may be speculated. Indirect mechanisms related with their capacity to quelate calcium in the extracellular environment might play a role. Calcium quelation, which is responsible for diminishing free calcium concentration, might inhibit MMPs activity since these enzymes are cation-dependent. Recently, it has also been speculated that the inhibitory action by TCs could be linked to a decreased level of collagenase mRNA, rather than a direct inhibition of the MMPs.[19,40,41]

Regardless the mechanism involved, it has been widely demonstrated that TCs can inhibit collagenase and/or the breakdown of collagen under a variety of conditions.[4,8,13,17] Studies in animal models of bone-deficiency disease showed that TCs were found to increase type I collagen synthesis.[42] Moreover, recent studies demonstrated that these agents can also increase collagen synthesis on soft tissues as well.[43–45] Evidence that a stable collagenous matrix is important for the progression of osteoblastic differentiation is well known[46,47] and provided, for instance, by studies showing that inhibition of collagen synthesis or its increased degradation leads to an impairment of osteoblastic cell behaviour.[48,49] In this way, the eventual modulation of the extracellular matrix by TCs might favour osteoblastic cell proliferation. Related to this, it is known that osteoblastic cells sense and respond to calcium ion in a time- and concentration-dependent manner, regarding proliferation and expression of several differentiation markers, including synthesis of collagen type I.[50] A variety of studies suggest that the calcium ion, most likely acting through the calcium sensing receptor (CaSR), is a key regulator of osteoblastic cell fate.[50,51] In this way, local fluctuations in the levels of calcium ion, potentially associated to the calcium quelation properties of TCs, might play a role in modulating osteoblastic cell behaviour.

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