Modulation of human osteoclastogenesis and osteoblastogenesis by lycopene

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Abstract

Lycopene is a lipid-soluble pigment that is mainly found in tomato. It is the carotenoid that presents the highest antioxidant potential, and due to that, it has been implicated in a decrease of the risk of several oxidative-stress-related disorders, such as cancer, inflammatory diseases and osteoporosis. Nevertheless, at the present, there is no detailed information about how lycopene affects bone metabolism. The aim of the present work was to characterize the cellular and molecular effects of lycopene on human osteoclast and osteoblast differentiation and function. It was observed that lycopene, at levels found in plasma after the ingestion of lycopene-containing products, decreased osteoclast differentiation but did not affect cell density/survival; calcium-phosphate resorbing ability was also decreased. On the other hand, osteoblast proliferation (via a decrease on apoptosis) and differentiation were increased in the presence of lycopene. The observed effects in both cell types appeared to be related to significant changes in MEK signaling pathway, but also in protein kinase C pathway in osteoclasts and NFkB signaling in osteoblasts. In conclusion, lycopene appears to promote an anabolic state of bone metabolism, stimulating osteoblastogenesis and inhibiting osteoclastogenesis, which may contribute to the promotion of a proper health status of bone tissue. This information might be relevant for the prevention and delay in the progression of osteolytic bone conditions.

Keywords: Lycopene; Osteoclastogenesis; Osteoblastogenesis; Bone metabolism; Cell differentiation

1. Introduction

Bone turnover is a continuous process that relies on a coordinated and complementary action of bone-synthesizing osteoblasts and bone-resorbing osteoclasts [1]. Among the stimuli that can modulate bone metabolism, oxidative stress appears to have a very important role. More precisely, it is known that the presence of reactive oxygen species (ROS) may decrease osteoblastogenesis [2] and increase osteoclast differentiation and function [3–9]. In this context, it is believed that ROS may be a key player in the pathogenesis of different pathological conditions, such as osteoporosis, with established positive correlations between the presence of excessive ROS and bone loss [5,10].

Lycopene is the carotenoid that displays the highest antioxidant behavior, particularly in the quenching of singlet oxygen [11,12], being more than twice that of β-carotene and about 10 times higher than that of α-tocopherol [11,13]. It is synthesized by many plants and microorganisms, and it is mainly found in red-colored fruits and vegetables, particularly in tomatoes (~80% of total lycopene consumption) but also in carrots, watermelon, pink grapefruit, rosehips and pink guava [12,14]. Chemically, lycopene is an open-chain hydrocarbon with 40 carbons and 11 conjugated double bounds in an all-trans configuration [11,14].

Mainly due to its high antioxidant potential, lycopene has been linked to a decreased risk of some chronic diseases in which oxidative damage may be an important factor, such as prostate and gastrointestinal cancers, cardiovascular disorders and osteoporosis [12,15–18]. In the latter case, it was observed that an increase in the consumption of lycopene caused a decrease in bone resorption levels in postmenopausal women [11,19–21], being claimed that this molecule may shift the bone metabolic equilibrium towards an increase in the anabolic state [22].

However, very little is known about the effects of lycopene at bone cellular and molecular levels. The few studies conducted about this subject have shown a decrease in the formation of rat osteoclasts [23] and an increase in human osteosarcoma SaOS-2 cell proliferation and differentiation [24]. The aim of the present study was to evaluate the effects of a wide range of lycopene concentrations on human primary osteoclast and osteoblast survival, proliferation and differentiation. A characterization of the underlying intracellular mechanisms involved in each cell type response to lycopene was also conducted.

2. Materials and methods

2.1. Osteoclastic cell cultures

Human peripheral blood mononuclear cells (PBMCs), used as osteoclastic precursors, were isolated from the blood of healthy donors 25–35 years old, after informed consent, as described previously [25]. For that, blood was diluted with PBS + 2 mM EDTA (1:1) and applied on top of Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400*g* (30 min), PBMCs were collected and washed twice with PBS + 2 mM EDTA. Cells were counted with a cytometer (Celltac MEK-5103). Typically, for each 100 ml of processed blood, about 70×10^6 PBMCs were obtained.

PBMCs were cultured in 96-well plates at 1.5×10^6 cells/cm^2 in α-MEM supplemented with 30% autologous human serum, 100 IU/ml penicillin (Thermo Fisher Scientific, MA, USA), 2.5 μg/ml streptomycin (Thermo Fisher Scientific, MA, USA), 2.5 μg/ml amphotericin B (Thermo Fisher Scientific, MA, USA) and 2 mM L-glutamine (Sigma-Aldrich, MO, USA). Cell cultures were maintained for 21 days in the absence (base medium, BM) or presence (M+R) of the osteoclastogenic inducers M-CSF (25 ng/ml, R&D Systems, MN, USA) and RANKL (40 ng/ml, Insight Biotechnology, Middlesex, UK) [26]. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C, and culture medium was replaced once a week.

Unstimulated and stimulated PBMC cultures were exposed to a wide concentration range of lycopene (Sigma-Aldrich, MO, USA), i.e., 5 nM to 50 μM. Lycopene was added 24 h after cell seeding, being present during the 21-day culture time, and renewed at each medium change. Cultures were assessed for total protein content; TRAP activity; presence of TRAP+ multinucleated cells; and presence of actin rings, vitronectin receptor (VNR) and calcitonin receptor (CTR).

Cultures treated with 500 nM lycopene, which was the minimum tested concentration that elicited a significant effect in osteoclastic behavior, were characterized for apoptosis, expression of osteoclastic genes, calcium phosphate resorbing ability and the involvement of several intracellular pathways on cell response. Cultures were assessed at days 14 and 21 as described below.

2.2. Osteoblastic cell cultures

Human mesenchymal stem cells bone marrow derived (HMSCs, Innoprot, Bizkaia, Spain) were used as osteoblastic precursors. Cells were cultured in α-MEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml ascorbic acid. When 70%–80% confluence was reached, cells were detached with 0.05% trypsin and 0.5 mM EDTA and seeded at 3.3×10^3 cells/cm^2 in 96-well plates. Cells were maintained for 21 days in the same culture medium, in the absence (−dex) or presence (+dex) of the osteogenic promotor dexamethasone (10 nM) [27]. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C, and culture medium was replaced once a week.

Unstimulated and stimulated HMSCs were exposed to a wide concentration range of lycopene, i.e., 5 nM to 50 μM. Lycopene was added 24 h after cell seeding, being present during the 21-day culture time, and renewed at each medium change. Cultures were assessed for total protein content, ALP activity and cell morphology.

Cultures treated with 500 nM lycopene, which was the minimum tested concentration that elicited a significant increase in osteoblastic behavior, were characterized for apoptosis, expression of several osteoblastic genes and the involvement of several intracellular pathways on cell response. Cultures were assessed at days 14 and 21 as described below.

2.3. Characterization of cellular responses

2.3.1. Protein quantification

Total protein quantification was performed by Bradford's method [28] using bovine serum albumin as a standard. Cells were washed with PBS, solubilized with 0.1 M NaOH and treated with Coomassie Protein Assay Reagent (Fluka, Milwaukee, WI, USA). After incubation for 2 min at room temperature, the 595-nm absorbance was determined in an enzyme-linked immunosorbent assay (ELISA) plate reader (Synergy HT, Biotek).

2.3.2. TRAP and ALP activities

TRAP and ALP activities were assayed by the *para*-nitrophenylphosphate (*p*NPP) hydrolysis method. Cells were washed twice with PBS and solubilized with 0.1% (v/v) Triton X-100. For TRAP activity, cellular extracts were incubated (1 h, 37°C) with 12.5 mM *p*NPP prepared in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8); for ALP activity, cellular extracts were incubated (30 min, 37°C) with 12.5 mM *p*NPP in alkaline buffer solution, pH 10.3. 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 and ALP activities were normalized with total protein content, and results were expressed as nmol/min.$\mu g_{protein}{}^{-1}$.

2.3.3. TRAP-positive multinucleated cells

PBMC cultures were washed twice with PBS and fixed with 3.7% formaldehyde for 10 min at room temperature. Afterward, cells were stained for TRAP with Acid Phosphatase, Leukocyte (TRAP) kit (cat. no. 387A, Sigma-Aldrich, MO, USA) according to manufacturer's instructions. For that, cells were incubated with 0.12 mg/ml naphtol AS-BI in the presence of 6.76 mM tartrate and 0.14 mg/ml Fast Garnet GBC for 1 h at 37°C in the dark. After incubation, cell layers were washed and stained with hematoxylin. TRAP-positive multinucleated cells were counted in a phase contrast microscope (Nikon TMS, Tokyo, Japan).

2.3.4. Actin, VNR and CTR staining

After 21 days of culture, cells were fixed with 3.7% formaldehyde for 10 min at room temperature. Afterward, cells were permeabilized with 0.1% Triton X- 100 for 5 min and stained for actin with 5 U/ml Alexa Fluor1 647-Phalloidin (Invitrogen) and, in the case of PBMC cultures, also for 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. Apoptosis quantification

Apoptosis was evaluated by caspase-3 activity quantification. For that, cells were washed twice with PBS and assessed for caspase-3 activity with EnzCheck Caspase-3 Assay Kit #2 (Molecular Probes, Eugene, OR, USA) 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 with the value obtained in the corresponding control (absence of lycopene) and were presented as a % of response compared to the control.

2.3.6. Osteoclast and osteoblast gene expression

Cell cultures were performed in six-well plates following the same experimental conditions described above. At day 21, gene expression was analyzed by quantitative real-time polymerase chain reaction (qPCR). The tested housekeeping genes were beta-glucuronidase (GUSB) and proteasome subunit beta type-6 (PSMB6). The genes whose expression was quantified were selected due to their importance for osteoclastogenesis or osteoblastogenesis. More precisely, the analyzed osteoclast-related genes were those coding for the differentiation and activation factors c-myc and c-src and for the osteoclastic enzymes TRAP, cathepsin K (CATK) and carbonic anhydrase 2 (CA2) [29]; the osteoblast-related genes were those coding for the bone matrix protein collagen type 1 (COL1), the osteoblastic enzyme ALP and the modulator of bone metabolism bone morphogenetic protein 2 (BMP2) [1]. 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, Finland) and random hexamers according to the manufacturer's instructions. Then, cDNA samples (2 ng) were amplified with a DyNAmo Flash SYBR green qPCR kit (Finnzymes, Finland) on a Rotor-Gene thermocycler (QIAGEN, Hilden, Germany). 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.

2.3.7. Calcium phosphate resorbing ability

PBMC cultures were performed on calcium-phosphate-coated culture plates (BD BioCoat Osteologic Bone Cell Culture Plates, BD Biosciences, NJ, USA) and maintained for 21 days in the absence or presence of 500 nM lycopene. Afterward, cells were bleached with 6% NaOCl and 5.2% NaCl, and the remaining calcium phosphate layers were visualized under phase contrast light microscopy (Nikon TMS, Tokyo, Japan). Resorption lacunae were identified, and total resorbed area was quantified with ImageJ 1.41 software. Results are presented as a % of resorbed area.

2.3.8. Intracellular signaling mechanisms

Osteoclastic and osteoblastic cell response to lycopene was characterized for the involvement of several common signaling pathways involved either in osteoclastogenesis [29,30] or osteoblastogenesis [27,31]. For that, PBMC and HMSC cultures were treated with the corresponding commercial signaling (Sigma-Aldrich, MO, USA) pathway inhibitor throughout the culture period (the inhibitors were renewed at each medium change). The tested pathways were MEK (inhibitor U0126, 1 μM), NFkB (inhibitor PDTC, 10 μM), protein kinase C (PKC) (inhibitor GO6983, 5 μM) and JNK (inhibitor SP600125, 10 μM). Cultures were assessed in 96-well plates for TRAP (PBMC cultures) and ALP (HMSC cultures) activities. The concentration of each inhibitor was selected based on previously published works with bone cells [32–35].

2.3.9. Quantification of signaling pathway proteins

The quantification of specific proteins involved in the tested signaling pathways was performed with ELISA kits following the manufacturer's instructions. MEK quantification was performed with the MEK1 ELISA Kit (cat. no. ab208988, Abcam, Cambridge, UK). NFkB quantification was performed with the NF kappaB p65 ELISA Kit (cat. no. ab176648, Abcam, Cambridge, UK). PKC quantification was performed with the PKC ELISA Kit (MyBioSource, San Diego, CA, USA). JNK quantification was performed with the JNK 1/2 ELISA Kit (cat. no. ab176646, Abcam, Cambridge, UK). Results are expressed as a % of the control (absence of lycopene).

2.4. Statistical analysis

Data presented in this work are the means of three independent experiments, with three replicates for each analysis. Statistical differences were assessed using an unpaired Student's *t* test with Bonferroni correction for multiple comparisons. All data

Table 1
Primers used on qPCR analysis of PBMC and HMSC cultures

Gene	5′ Primer	3′ Primer
GUSB	5′-TGCAGCGGTCTGTACTTCTG-3′	5′-CCTTGACAGAGATCTGGTAATTCA-3′
PSMB6	5′-GCCGGCTACCTTACTACCTG-3′	5′-AAACTGCACGGCCATGATA-3′
c-myc	5′-TACCCTCTCAACGACAGCAG-3′	5′-TCTTGACATTCTCCTCGGTG-3′
c-src	5′-AAGCTGTTCGGAGGCTTCAA-3′	5′-TTGGAGTAGTAGGCCACCAG-3′
TRAP	5′-ACCATGACCACCTTGGCAATGTCTC-3′	5′-ATAGTGGAAGCGCAGATAGCCGTT-3′
CATK	5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′	5′-CTTGCATCAATGGCCACAGAGACAG-3′
CA2	5′-GGACCTGAGCACTGGCATAAGGACT-3′	5′-AAGGAGGCCACGAGGATCGAAGTT-3′
COL1	5′-TCCGGCTCCTGCTCCTCTTA-3′	5′-ACCAGCAGGACCAGCATCTC-3′
ALP	5′-ACGTGGCTAAGAATGTCATC-3′	5′-CTGGTAGGCGATGTCCTTA-3′
BMP-2	5′-GACGAGGTCCTGAGCGAGTT-3′	5′-GCAATGGCCTTATCTGTGAC-3′

are expressed as the mean ± standard deviation/error. For values of $P\leq.05$, differences were considered statistically significant.

2.5. Ethics

This study was approved by the institutional ethics committee.

3. Results

3.1. Effects of lycopene on osteoclastogenesis

Cell cultures performed in BM and maintained in the absence of lycopene exhibited a low total protein content, which decreased throughout the culture period (Fig. 1A, upper panel). The presence of lycopene did not significantly affect cell response. In M+R condition, total protein content was higher but still decreased during the time of culture (Fig. 1A, lower panel). Supplementation with lycopene, particularly at high doses, elicited a decrease, though not significant, on total cell protein content. Regarding TRAP activity, cell response in BM was low, increasing over the culture period (Fig. 1B, upper panel). At low concentration, lycopene did not affect TRAP activity; however, at doses ≥500 nM, the values were significantly decreased (~20%–46%). Treatment of PBMC cultures with the osteoclastogenic enhancers caused an increase in TRAP activity (Fig. 1B, lower panel). Once again, lycopene at concentrations ≥500 nM promoted a significant decrease on cell response (~31%–42%). In either BM or M+R, the amount of TRAP+ multinucleated cells in the different experimental conditions (Fig. 1C) followed a similar pattern to that observed for TRAP activity.

It was possible to observe cells with actin rings and expressing VNR or CTR, in all tested conditions (Fig. 2A). Globally, either in BM or M+R, the presence of lycopene caused a decrease in the amount of those cells.

Having established a dose–response curve for PBMC cultures treated with lycopene in both BM and M+R conditions, it was also intended to evaluate if this carotenoid modulates apoptosis by means of caspase-3 activity quantification (Fig. 2B). No significant differences were observed between the control and cell cultures treated with lycopene in both BM and M+R conditions. Cells were also assessed for the expression of some osteoclast-related genes, namely, c-myc, c-src, TRAP, CATK and CA2 (Fig. 2C). As observed, lycopene elicited a decrease in the expression of all of the tested genes (~12%–44% and 18%–50% lower than the control in BM and M+R, respectively).

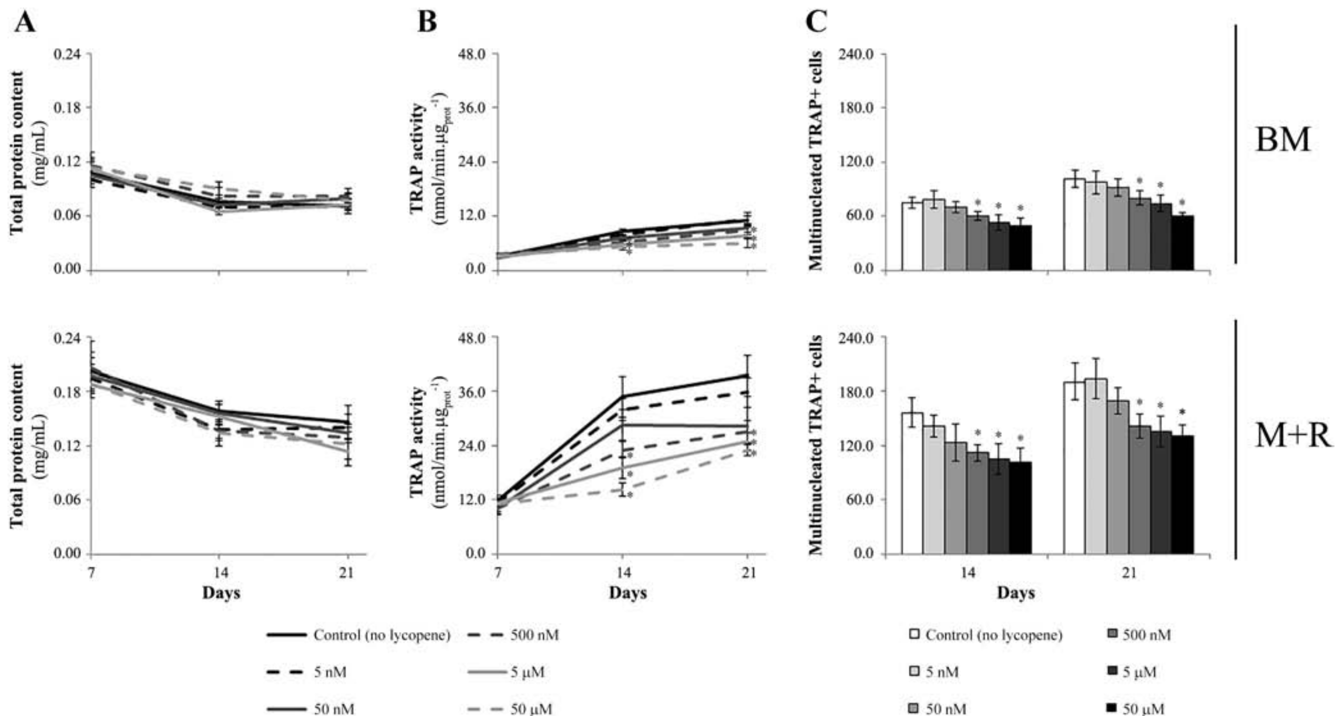

Fig. 1. Dose–response curves of PBMC cultures treated with lycopene and maintained in the absence (BM) or presence (M+R) of M-CSF and RANKL. (A) Total protein content. (B) TRAP activity. (C) Presence of TRAP+ multinucleated cells. *Significantly different from the control.

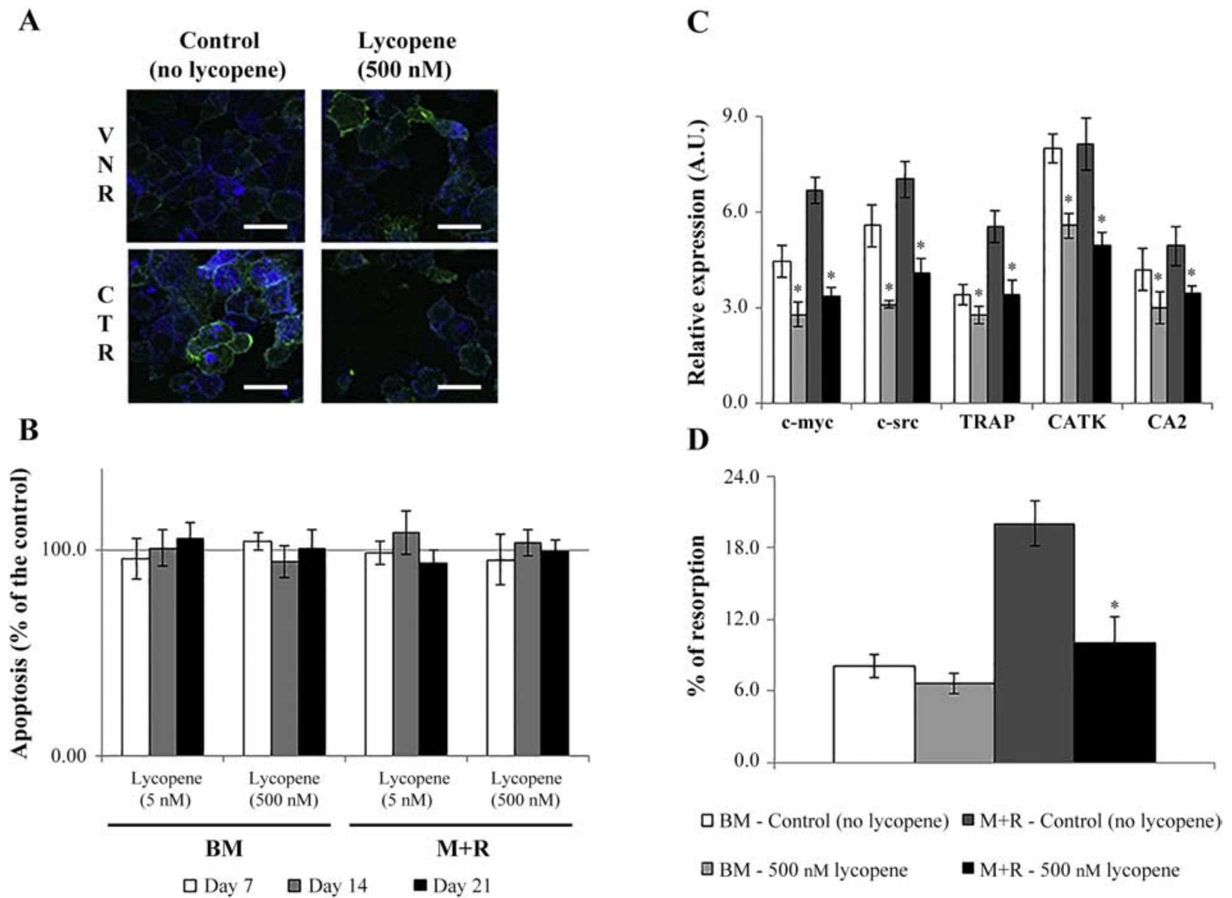

Fig. 2. Effects of 500 nM lycopene on PBMC cultures maintained in the absence (BM) or presence (M+R) of M-CSF and RANKL. (A) Presence of cells with actin rings (blue) and expressing VNR and CTR (green); white bars represent 150 μm. (B) Caspase-3 activity. (C) Expression of osteoclast-related genes. (D) Calcium phosphate resorbing ability. *Significantly different from the control.

Regarding calcium phosphate resorbing ability (Fig. 2D), lycopene did not significantly affect cell response in BM condition, while decreasing it significantly in M+R condition (~50%). This inhibitory effect of lycopene may be linked to a direct decrease in the resorbing function of the formed osteoclasts or may be a consequence of a decreased amount of osteoclastic cells.

After the characterization of lycopene effects on osteoclastogenesis, the involvement of several intracellular signaling pathways on the observed cellular behavior was characterized (Fig. 3). It was observed that none of the tested inhibitors had a significant effect on total protein content (Fig. 3A). In BM (and absence of lycopene), TRAP activity (Fig. 3B) was significantly lowered by the presence of U0126 (~64%), GO6983 (~46%) and SP600125 (~49%) and completely abolished by PDTC. Supplementation with lycopene caused an increase in the inhibition promoted by U0126 (~82%) and SP600125 (~60%). In M+R, once again, U0126 (~43%), GO6983 (~35%) and SP600125 (~41%) elicited a decrease in cell response, and PDTC totally inhibited TRAP activity. In the presence of lycopene, the decrease observed in cell cultures supplemented with U0126 (~77%) and SP600125 (~53%) was significantly higher than in the absence of the carotenoid. In order to go further in the characterization of the effects of lycopene on the tested signaling pathways, it was conducted a quantification of specific proteins of each pathway (Fig. 3C). It was observed that either in BM or M+R, lycopene promoted an increase in MEK and PKC proteins (~22-55%).

3.2. Effects of lycopene on osteoblastogenesis

Since osteoblasts are proliferative cells, which establish extensive cell-to-cell contacts, the relative cell density present in each experimental condition was inferred from total protein content. In control conditions, total protein content was similar in both the absence or presence of dexamethasone (−dex and +dex, respectively) (Fig. 4A). Supplementation with lycopene significantly increased cell response for concentrations ≥500 nM (~18%–22% and ~19%–25% in −dex and +dex conditions, respectively). Regarding ALP activity (Fig. 4B), HMSC cultures maintained in −dex conditions exhibited low values, which were significantly increased by lycopene at concentrations ≥500 nM (~15%-−19%). In an osteogenic medium (+dex), HMSCs revealed higher ALP activity, and supplementation with lycopene (≥500 nM) further increased that behavior (~22%–31%).

In all tested conditions, cell cultures exhibited well-spread cells, with the expected morphology, i.e., elongated cells, with cytoplasmic extensions and cell-to-cell contacts (Fig. 5A). Cell cultures performed in the presence of lycopene revealed higher amounts of osteoblastic cells, in line with what was observed for total protein quantification.

In order to understand if the effects of lycopene on cell density (inferred from total protein content) were due to differences on apoptosis, caspase-3 activity was quantified (Fig. 5B). It was observed that in either −dex or +dex condition, lycopene caused a significant decrease on cell response. Also, the expression of some osteoblast-

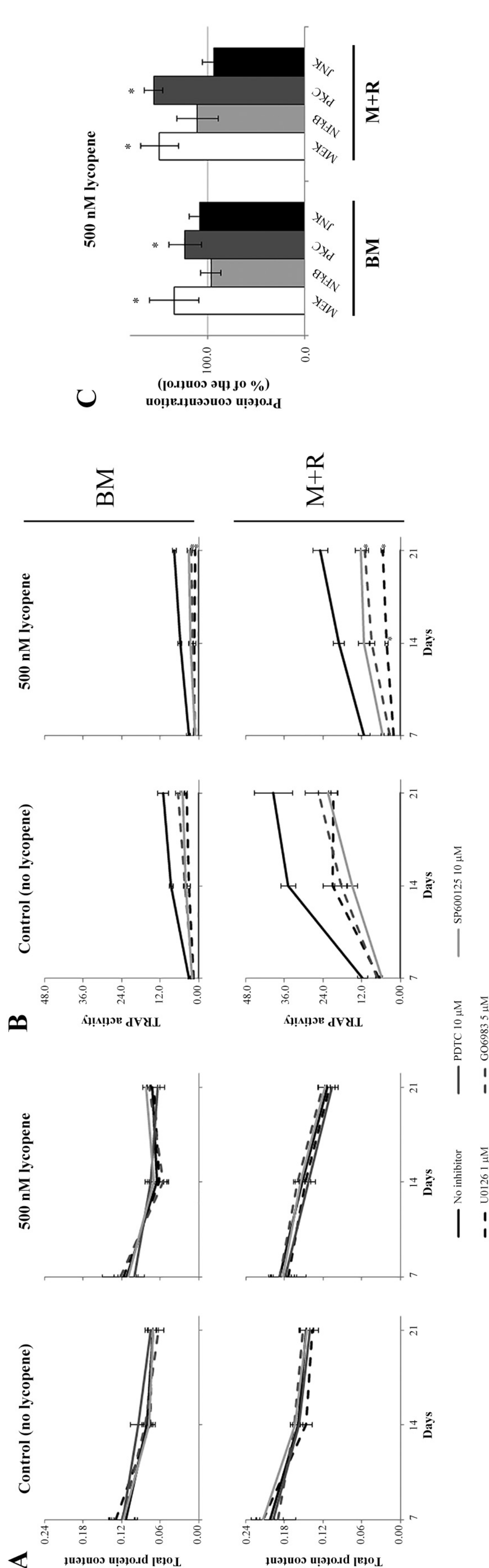

Fig. 3. Modulation of MEK, NFkB, PKC and JNK signaling pathways by lycopene in PBMC cultures. Cell response was evaluated by (A) total protein content, (B) TRAP activity and (C) specific signaling pathway proteins quantification. *Significantly different from the control.

related genes was increased by this molecule (Fig. 5C), particularly in osteogenic conditions, with values ~40% and 47%–101% higher than the control (−dex and +dex, respectively). The only exception was BMP2, whose expression was not significantly affected by lycopene in −dex cell cultures.

In order to gain some insights about the intracellular mechanisms modulated by lycopene on HMSC, cell cultures were treated with different signaling pathway inhibitors and assessed for ALP activity (Fig. 6). Despite the absence or presence of dexamethasone, total protein content was not significantly affected by the signaling pathway inhibitors (Fig. 6A). Cell cultures maintained in −dex revealed a decrease on ALP activity (Fig. 6B), prompted by the different inhibitors (~27%–52%). The highest inhibition was achieved in the presence of PDTC. Following supplementation with lycopene, a similar pattern of cell response was observed, with one main difference: in the presence of U0126, ALP activity was not decreased. In +dex conditions, all the tested inhibitors elicited a decrease on cell response (~26%–44%), but once again, in the presence of lycopene, U0126 supplementation did not affect ALP activity. Also, the inhibition promoted by PDTC was lower in the presence of lycopene (~14%). Regarding the signaling pathway protein levels, it was observed that in −dex conditions, MEK concentration was decreased by lycopene (~33%), while in +dex, the decrease affected both MEK and NFkB proteins (~22%–40%).

4. Discussion

Bone metabolism is highly dynamic, being continuously modulated by numerous intrinsic and extrinsic factors. Among the latter, diet is one of the most important factors that may contribute to a proper, or to a disrupted, bone structure and function [36]. Nutrients like vitamin D and calcium are well studied in respect to their effects on bone matrix production and mineralization [37,38]. However, other nutritional factors may also be implied in the process. For instance, it is known that oxidative stress is involved in the pathogenesis of many chronic diseases, like cardiovascular diseases, diabetes and cancer [15,16]. In addition, bone metabolic disorders are also related to the oxidative levels achieved in bone microenvironment. In this context, antioxidants appear as key regulators of bone cellular activities, acting on both osteoclastogenesis [39,40] and osteoblastogenesis [2], which means that antioxidant depletion may have a negative impact on bone mass.

In line with this, it was reported that a proper diet rich in fruits, vegetables, nuts and seeds is associated with higher values of bone mineral density (BMD) [41,42], with a positive association being observed between osteoporosis and oxidative stress markers [2,4,43]. Also, increased levels of the oxidative stress markers 8-isoprostaglandin F2α and malondialdehyde, as well as decreased levels of the antioxidant enzymes superoxide dismutase and glutathione peroxidase, were correlated with low BMD values and alkaline phosphatase activity [4,7,43].

Among the dietary antioxidants, carotenoids represent a heterogenous though very important group, which is present mainly in fruits and vegetables. The main carotenoids ingested by humans are α- and β-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene, with the latter being the most potent antioxidant of the group with twice the antioxidant capacity of β-carotene and 10 times that of α-tocopherol [11,44]. A positive association between fruit intake and BMD was observed in different studies conducted with individuals belonging to different populations [45–47].

Mainly due to its high antioxidant potential, high plasma levels of lycopene were demonstrated to correlate with a decrease in the risk of prostate, gastrointestinal, cervical and pancreatic cancers, and also in myocardial infarction [15,48]. Lycopene has also been linked to a healthier bone tissue. In a study with postmenopausal Canadian

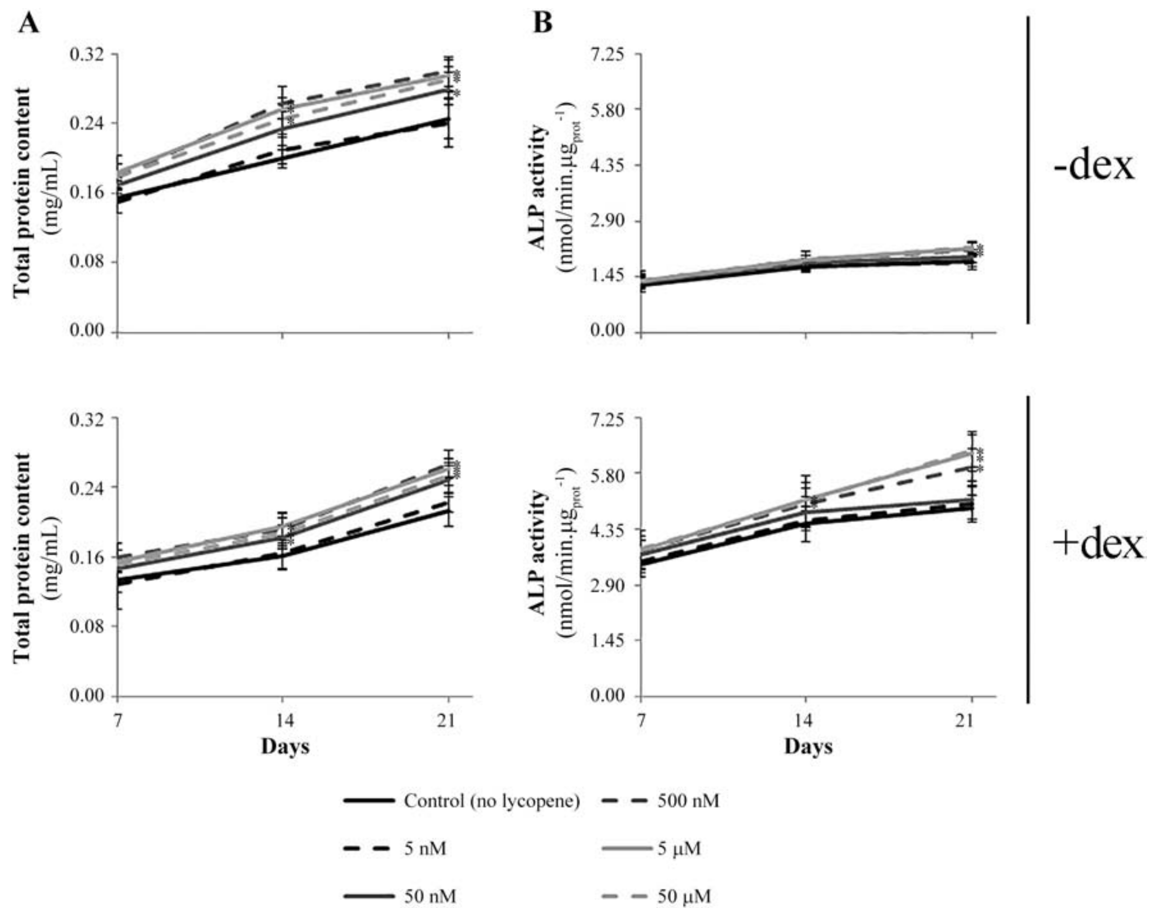

Fig. 4. Dose–response curves of HMSC cultures treated with lycopene and maintained in the absence (−dex) or presence (+dex) of dexamethasone. (A) Total protein content. (B) ALP activity. *Significantly different from the control.

women, a decrease in N-telopeptides of collagen type I following lycopene consumption was observed [19]. Several other human studies revealed an increase in BMD in different anatomical regions, associated with an increase in lycopene consumption, both in men and in women [12,17,19–21,49–52]. In rodents, the daily administration of lycopene revealed to be protective against ovariectomy-induced bone loss, promoting an increase in bone anabolic markers, such as serum ALP [53,54]. In an ovariectomized mice model, it was observed that lycopene revealed a bone protective effect similar to alendronate, improving bone biomechanical properties [55]. These findings highlight the need to characterize the cellular and molecular effects of lycopene on bone cells in order to understand the real effects that lycopene may exert on bone metabolism and, consequently, on bone health.

The present study aimed to characterize the cellular and molecular effects of lycopene on human primary osteoclasts and osteoblasts, an issue not addressed previously. To do that, both osteoclast and osteoblast precursor cells were maintained in the absence or presence of osteoclastogenic and osteogenic activators, respectively, and were treated with lycopene. Although there are significant differences in the plasma levels of lycopene found in humans, since the published values range between of 0.160 and 1.97 μM [19,21,22,56], in the present work, the wide range of tested concentrations is expected to cover the normal lycopene levels in the majority of the situations. In this study, lycopene showed the ability to directly act on human osteoclasts and osteoblasts. Regarding the former cell type, lycopene at concentrations ≥500 nM (a value that may be representative of normal lycopene plasma levels) caused a decrease on osteoclast differentiation, while not affecting cell density, either in nonstimulated or in stimulated osteoclast precursor cell cultures. It is important to highlight that the starting biological material for osteoclast cultures was a heterogeneous population (PBMC), composed by different cell types that, as described previously [57], contain other types in addition to the CD14+ osteoclast precursors, which may support a low osteoclastogenic response even in the absence of any exogenous osteoclastogenic stimuli. Osteoblastogenesis was also modulated by lycopene, which promoted a significant activation of osteoblast cell proliferation and differentiation at levels ≥500 nM. In this case, lycopene had a higher effect in the presence of an osteogenic enhancer (dexamethasone). These results are in line with the few published studies about this issue. More precisely, in the unique study about the effects of lycopene on osteoclasts, a decrease on osteoclastogenesis was observed in rat bone marrow cell cultures treated with 100 nM lycopene due to a decrease in the production of reactive oxygen species [23]. A down-regulation on RANKL production in lycopene-treated mice was also observed [55]. In studies conducted with human osteoblast-like osteosarcoma SaOS-2 cells, it was observed that lycopene at 100 nM and 1 μM caused an increase in cell proliferation and osteogenic differentiation [24]. In female rats, the ingestion of lycopene elicited an increase in serum bone-type ALP [54]. In another work, it was demonstrated that lycopene elicited an increase in COL1 expression [55]. In a work with mice mesenchymal stem cells, it was observed that, after hypoxia, cell viability (assessed by the MTT assay) was increased in the presence of lycopene at doses ≥20 μM [54]; in cell cultures maintained in the presence of oxygen, lycopene had no significant effect. Furthermore, lycopene at 20 μM caused a decrease

Fig. 5. Effects of 500 nM lycopene on HMSC cultures maintained in the absence (−dex) or presence (+dex) of dexamethasone. (A) Presence of cells stained for actin (blue); white bars represent 30 μm. (B) Caspase-3 activity. (C) Expression of osteoblast-related genes. *Significantly different from the control.

on apoptosis. Effects on osteoblast and osteoclast apoptosis were also observed in an ovariectomized mice model [55]. Taken together, the present work and the few published reports suggest a potential anabolic effect of lycopene on bone cellular metabolism.

Having established how lycopene modulates both osteoclastogenesis and osteoblastogenesis, it was intended to go further in this issue, namely, at the level of the intracellular mechanisms involved in the observed cell behavior. For that, cell cultures were treated with the minimum lycopene concentration that was able to significantly affect osteoclast and osteoblast development (500 nM), and were further supplemented with commercial inhibitors of different signaling pathways. None of the tested inhibitors displayed a significant effect on cell density (inferred from total protein content), which suggests that there was no significant cytotoxicity. Nevertheless, it was observed that, independently of the presence of M-CSF and RANKL, lycopene promoted an up-regulation of MEK and PKC pathways. In the case of osteoblastic cell cultures, in the absence of dexamethasone, MEK pathway was down-regulated by lycopene; in the presence of the osteogenic promotor, MEK and NFkB signaling pathways appear to lose relevance in cell cultures treated with lycopene. In order to evaluate if there was indeed a direct modulation of lycopene on those pathways, a quantification of specific proteins of each signaling pathways was conducted. The results were in line with the observed behavior of cells in the presence of the inhibitors, which points to a direct effect of lycopene on MEK and PKC pathways during osteoclastogenesis and MEK and NFkB during osteoblastogenesis.

Previously, it was demonstrated that oxidative stress is an activator of bone resorption and that its mechanisms of action may involve the activation of NFkB signaling pathway, as well as other intracellular processes [58–60]. Considering that lycopene displays a robust antioxidant capacity, this may represent one important target of this molecule and may explain some of the present results. Another important issue relates with MEK pathway because it was previously observed that this pathway is also affected by oxidative stress [61], and in osteoclast and osteoblast cell cultures, MEK was modulated by lycopene. Nevertheless, one cannot exclude the involvement of other important processes in the observed cell responses, such as stimulation of gap junction communications, induction of the production of detoxifying enzymes [62] and connexin 43 [63], and actions on cell cycle checkpoints [64]. Since intercellular communications through gap junctions are key events of normal osteoclast and osteoblast metabolism and, consequently, on bone remodeling [65], and lycopene may modulate gap junction communication [66], it is tempting to suggest that this may also represent a potential target of action of lycopene on bone cells.

Taken together, the present data point to an anabolic effect of lycopene on bone metabolism and, consequently, a potential positive effect on bone structure, strength and function. However, in order to obtain the desired effects, high levels of lycopene must be achieved. Thus, a satisfactory ingestion of lycopene must occur since serum lycopene positively correlates with total fruit and vegetable intake [19,22,46]. Understanding how bone tissue responds to nutritional

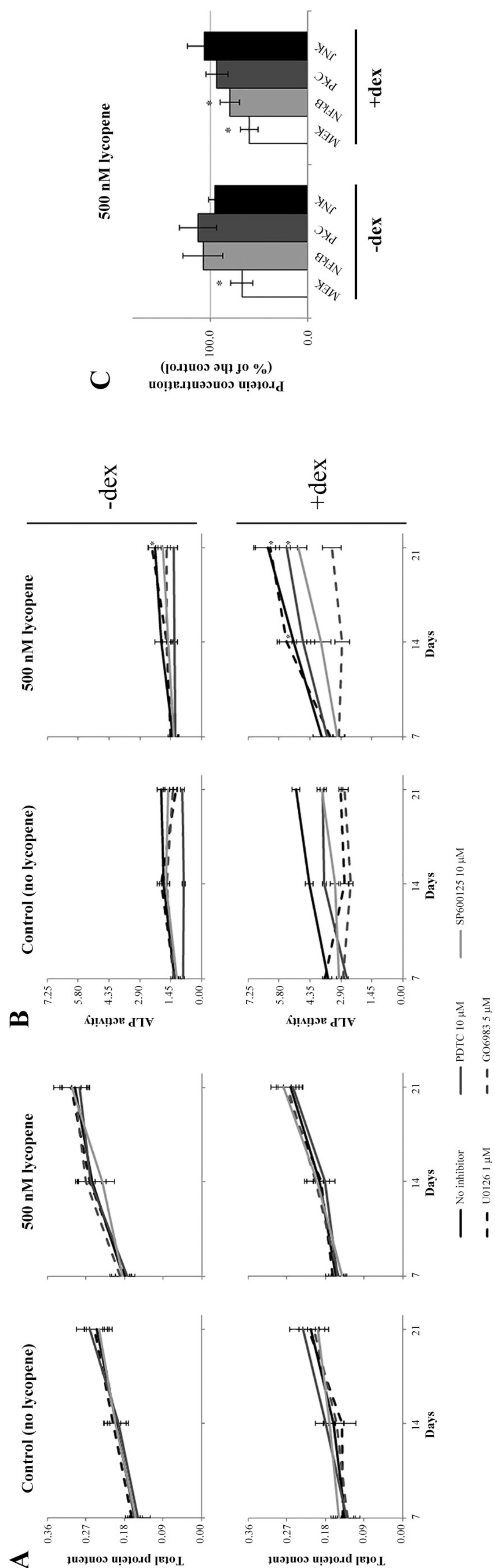

Fig. 6. Modulation of MEK, NFkB, PKC and JNK signaling pathways by lycopene in HMSC cultures. Cell response was evaluated by (A) total protein content, (B) ALP activity and (C) specific signaling pathway proteins quantification. *Significantly different from the control.

approaches aiming to preserve bone mass and strength is a very important issue since it may provide good results in the maintenance of a healthier state of bone tissue and may reveal important strategies to face with worldwide bone disorders like, for example, osteoporosis.

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