

Lycopene nanoparticles promotes osteoblastogenesis and inhibits adipogenesis of rat bone marrow mesenchymal stem cells

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Abstract. – OBJECTIVE: Lycopene is a carotenoid and antioxidant with potent singlet oxygen quenching ability that reduces oxidative stress and promotes bone health. However, the cellular mechanisms by which lycopene influences bone metabolism are not known.

MATERIALS AND METHODS: The present study investigated the effects of lycopene nanoparticles on the differentiation of rat bone marrow-derived mesenchymal stem cells into osteoblasts or adipocytes.

RESULTS: In osteogenic medium, lycopene supplementation dose-dependently enhanced osteoblast differentiation, as evidenced by the transcription of *Alpl*, *Runx2*, *Col1a1*, *Sp7*, and *Bglap*, higher alkaline phosphatase activity, osteocalcin secretion and extracellular matrix mineralisation seen with Alizarin red S staining, and increased haem oxygenase levels. By contrast, lycopene in adipogenic medium inhibited adipocyte differentiation evidenced by decreases in the transcription of *Tnfsf11*, *Tnfrsf11b*, *Pparg*, *Lpl*, and *Fabp4* and reduced fat accumulation observed by Oil Red O staining.

CONCLUSIONS: Lycopene nanoparticles may promote bone health and are considered as a potential candidate for the prevention and/or treatment of bone loss conditions.

Key Words:

Lycopene, Mesenchymal stem cells, Differentiation, Adipocyte, Osteoblast, Bone loss.

Introduction

The loss of bone strength and mass with age is a determinant of osteoporosis and the risk of fracture¹. This results in considerable morbidity as well as mortality, such as following hip fractures². Genetics along with metabolic, endocrine, and lifestyle factors contribute to such bone loss, characterized by insufficient osteoblastogenesis during bone remodeling in association with enhanced osteoclastogenesis³. The decreased osteoblastogenesis and increased adipogenesis in bone marrow jeopardize bone health and integrity, resulting in age-related bone loss⁴. The bone loss in case of osteoporosis, diabetes, and aging, may result from overproduction of reactive oxygen species (ROS)⁵. Consequently, this inhibits the differentiation and proliferation of osteoblasts, damaging many cellular components and impairing osteoblastic activity⁶. The ROS signaling increases osteoclast differentiation and activity, leading to higher bone resorption^{7,8} and thus bone weakness and fragility⁹.

As osteoblasts and adipocytes both derived from multipotent mesenchymal stem cells (MSCs) in bone-marrow¹⁰, there is reciprocal relationship between osteogenesis and adipogenesis¹¹. During aging, a decrease in bone formation is predominated by increases in marrow adiposity that accompany

osteoporosis^{12,13}. The differentiation of pluripotent bone marrow MSCs (BMSCs) into osteoblasts or adipocytes is influenced by oxidative stress, nutrients, and metabolic and endocrine signals, as well as by several drugs; thus, stimuli that induce osteoblastogenesis reciprocally inhibit adipogenesis¹⁴.

To promote bone healing or treat osteoporosis, a reduction in ROS production *via* antioxidants is considered an effective approach¹⁵. One robust antioxidant is the lycopene, a carotenoid with potent singlet oxygen quenching activity naturally found in tomatoes¹⁶ and is the most predominant carotenoid in human plasma¹⁷. *In vitro*, the lycopene was shown to inhibit tartrate-resistant acid phosphatase production and the ROS secretion. This leads to the diminishing the differentiation of bone marrow cells into osteoclasts¹⁸. It was previously reported¹⁸ that, lycopene stimulated osteoblastogenesis and inhibited osteoclastogenesis in cultured human cells. In addition, lycopene prevented the bone loss and restored bone strength in ovariectomized rats^{19,20}. A significant improvement in bone mass, as well as in the geometric and biomechanical properties of bone, was reported²⁰. It was evident that, the healthy postmenopausal women who administered diets low in lycopene had markedly lower circulating levels of superoxide dismutase and catalase enzymes²¹. In addition, the diet supplemented with lycopene reduced the levels of oxidative stress markers and N-terminal telopeptides of collagen type 1 in healthy postmenopausal women²².

Heme oxygenase (HO)-1, the rate-limiting enzyme in hem metabolism, is cytoprotective and upregulated by oxidative stress or cellular injury^{23,24}. In macrophages, the activation of HO-1 and its signal pathways is anti-inflammatory²⁵. HO-1 expression is stimulated by lycopene in cisplatin-induced nephrotoxicity⁶, and in rats with diethylnitrosamine-induced hepatocarcinogenesis²⁷, leading to an increased resistance to oxidative stress-mediated damage. Moreover, HO-1 is involved in differentiation of BMSCs²⁸.

This study investigates how the lycopene affects osteoblastogenesis and adipogenesis using *in vitro* model of rat BMSCs under conditions favoring either osteoblast or adipocyte differentiation, as well as on the expression of HO-1.

Materials and Methods

Reagents

The reagents used in this study were from Sigma-Aldrich Co. (St. Louis, MO, USA) unless oth-

erwise indicated. Culture media, fetal bovine serum, and streptomycin, and all other cell culture materials, supplies, and reagents were purchased from Corning Inc. (Corning, NY, USA), HyClone (Logan, UT, USA), Gibco and Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Nanopure water was obtained from Macron Chemicals (Charlotte, NC, USA).

Experimental Animals

Male Wistar rats (~5 weeks old, weighing 150-160 gm) from the animal house at King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia, were housed separately in a controlled environment (22°C with a 12-h light/dark cycle) and given *ad libitum* access to water and standard rodent chow as described previously²¹. Animals were selected carefully and examined well prior involvement in the study. Bone marrow stromal cells were isolated from these animals by using a protocol approved by the ethics committee of the Centre for Excellence in Osteoporosis Research. The study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee and the international Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23).

Lycopene Nanoformulation

Polylactic-co-glycolic acid nanoparticles [copolymer lactide/glycolide, 50:50 (MW 40,000-75,000 g/mol)] encapsulating lycopene were prepared by double emulsion-solvent evaporation as described previously²⁹. Briefly, 500 ml each of stock solutions of 80 mg/ml polylactic-co-glycolic acid polymer and 10 mg/ml lycopene³⁰, both in dichloromethane (CTL Scientific Supply Co., Deer Park, NY, USA), were mixed by vortexing.

To obtain primary emulsions, this solution was first mixed with 200 ml of phosphate-buffered saline (PBS) by intermittent sonication [2-3 times, 30 sec each time, at 13,000 rpm (Ultra-Turrax T25 basic; Ika Works, Wilmington, NC, USA)], and then, sonicated for 30 sec in 2 ml of 1% (w/v) polyvinyl alcohol (MW 30,000-50,000 g/mol) solution. The obtained oil-in-water emulsion was then added to 40 ml of 1.0% polyvinyl alcohol solution and mixed for 30 min with constant magnetic stirring. The dichloromethane was then evaporated by using a rotatory evaporator (at low pressure and maintained at 37°C), and the solution was dialyzed with a 10-12 kDa dialysis membrane on a Millipore-Labscale TFF system (Millipore Corp.,

Burlington, MA, USA) against water for 24 h to remove the impurities and residual solvents. The entire solution was lyophilized and re-dispersed for further use. Validation studies were performed on particle size and size distribution, and the nanoparticle morphology was characterized along with controlled release and antioxidant activity, as described previously²⁹. The concentrations of lycopene used in the present study are within the range of published human plasma values^{16,24}.

Isolation of Rat BMSCs

Rats were euthanized by decapitation and both long bones (tibias and femurs) were immediately removed and cleaned of any attached muscles and tissues under aseptic conditions. After removing the epiphyses, bone marrow plugs were flushed with Dulbecco's Modified Eagle Medium/F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin using a 23-G needle. The cell mixture was strained to remove debris, and the plugs were passed through a 76- μ m wire mesh filter to ensure a single-cell suspension³¹. Six milliliters of cells (5×10^5 cells/ml) per 100-mm culture dishes (Nunc, Denmark) were cultured for at 37°C for 72 h in a humidified atmosphere with 5% CO₂. The cells were trypsinized and sub-cultured when they reached 80% confluency. The purity of sub-cultured cells (1×10^6 cells) was determined by flow cytometry (BD FACS Calibur system; BD Biosciences, San Jose, CA, USA) with PE-labelled CD45, CD90, or CD11b, FITC-labelled anti-CD44, and isotype controls (all purchased from BD Biosciences, NJ, USA). The cultured cells identified as MSCs were used to study the optimal lycopene concentration that improves and promotes osteogenic differentiation.

Induction of Osteoblastic and Adipogenic Differentiation in Rat BMSCs

Rat BMSCs (1×10^4 cells) were cultured at 37°C with 5% CO₂ in 24-well plates with either osteogenic medium (OM) containing 10 mM β -glycerophosphate, 0.1 nM dexamethasone, and 50 mg/ml ascorbic acid phosphate or adipogenic medium (AM) containing 5 mg/ml insulin, 50 μ M indomethacin, 10 nM dexamethasone, and 0.5 mM isobutylmethylxanthine; media were refreshed every 3 days. Lycopene was supplemented at concentrations of 0, 1.25, 2.5, and 5.0 μ M concurrent with the differentiation process. The control medium (CM) is OM or AM but without lycopene.

Cell Viability

Cell viability was examined by MTT Kit (MTT Kit; Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's guidelines. Briefly, cells were incubated in 96-well plates (3×10^3 cells/well in OM) for 7 and 14 days (supplemented with lycopene at 0, 2.5, 5, 10, 15 and 20 mM). Following the incubation period, the cells were incubated with 10% MTT (5 mg/ml) in culture medium for 4 h (in a humidified 5% CO₂ at 37°C). The medium was then aspirated from each well, and 1 ml of isopropanol (0.04 N HCl in isopropanol) was added to each well. Optical density (OD) was determined at 570 nm using a microplate reader (Bio-Teck Instruments, Winooski, VT, USA). Three independent experiments were performed for each time interval.

Determination of Alkaline Phosphatase Activity (ALP) in Osteoblasts

Osteoblasts were harvested and lysed by sonicating for 15 sec in PBS containing 0.1% Triton X-100 and 2 mM MgCl₂. ALP protein content and activity in the supernatant were determined with commercially available kits and reagents (Ortho-Clinical Diagnostics, Johnson's & Johnsons Co., New Brunswick, NJ, USA). The ALP results were normalized to protein concentration.

Osteocalcin Secretion Determination

To examine the effects of lycopene on osteocalcin secretion by cultured r-BMSCs (as part of osteoblastic function), the levels of osteocalcin were determined. Thus, osteocalcin released by cultured cells into media (in 0.5ml of media) were collected during 3-12 days of culture. Osteocalcin levels were measured with commercially available Rat-Mild™ Osteocalcin EIA Kit (Immunodiagnostic Systems Ltd., Fountain Hills, USA).

Alizarin Red S Staining and Calcium Deposition Assay

Alizarin red S staining was performed as previously described³² with minor modifications. Cells grown in OM for 21 days and washed thrice with PBS, fixed with 4% formaldehyde for 30 min, and rinsed thrice with distilled H₂O for 5 min. The cells were then stained with 1% Alizarin red S in 2% ethanol (pH 4.0) for 5 min at room temperature, washed thrice with distilled H₂O. Then, they were incubated for 2 min with 70% ethanol. After the cells were washed with distilled H₂O, calcified nodules which appeared as bright red color were photographed, and areas

with mineralized nodules stained at different intensities were scanned.

Oil Red-O Staining of Adipose Cells

Cells cultured in AM were fixed with 4.0% formaldehyde for 10 min, rinsed with distilled H₂O, and then incubated for 20 min with Oil Red O solution at room temperature³³. The cells were then washed with 85% propylene glycol for 3 min, rinsed with distilled H₂O, and mounted on slides with an aqueous mounting medium. Oil Red O-positive cells were quantified under light microscopy.

RNA Extraction and Real-Time Quantitative PCR

Total cellular RNA was obtained from cultured cells by using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and cDNA was synthesized from 1mg of DNase-treated total RNA with an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. Real-time quantitative PCR was performed on triplicate reactions using SYBR Green (SA Biosciences, Valenica, CA, USA) with an Ap-

plied Biosystems 7900 HT Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers listed in Table I were designed based on sequences in the GenBank database or published species-specific sequences for genes encoding alkaline phosphatase (*Alpl*), runt-related transcription factor 2 (*Runx2*), collagen type I, alpha 1 (*Colla1*), osterix (*Sp7*), osteocalcin (*Bglap*), receptor activator for nuclear factor κB ligand (*Tnfsf11*), osteoprotegerin (*Tnfrsf11b*), peroxisome proliferator-activator receptor gamma 2 (*Pparg*), lipoprotein lipase (*Lpl*), fatty acid-binding protein (*Fabp4*), heme oxygenase-1(*OH-1*) and glyceraldehyde phosphate dehydrogenase (*Gapdh*). All the analyses were performed in triplicates. All results were examined by using the comparative cycle number at threshold method with expression levels relative to *Gapdh*.

Western Blot Analysis

To measure the HO-1 expression, cell culture protein extracts were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat milk for 2 h at room temperature, the membranes were incubated at

Table I. Primer sequences used in this study.

Gene	Primer sequences (5'→3') (forward/reversed)	Product size (bp)
Alkaline phosphatase (<i>Alpl</i>)	CCAACGTGGCTAAGAATGTCATC TGGGCATTGGTGTGTACGTC	175
Runt-related transcription factor 2 (<i>Runx2</i>)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Collagen, type 1, alpha 1 (<i>Colla1</i>)	CGCTGGCCCCAAAGGATCTCCTG GGGTCCGGGAACACCTCGCTC	263
Osterix (<i>Ost</i>)	AGCCAGAAGCTGTGAAACCTC AGCTGCAAGCTCTCCATAACC	163
Osteocalcin (<i>Sp7</i>)	CCATGAGAGCCCTCACACTCC GGTCAGCCAACCTCGTCACAGTC	258
Receptor activator for nuclear factor κB ligand (<i>Tnfsf11</i>)	AATATCAGAAGATGGCACTC GATGTGCTGTGATCCAACG	187
Osteoprotegerin (<i>Tnfrsf11b</i>)	GCGCTAACCTTGAGATAGAGTTCTG TGTTTTCTACAGGGTGCTTTAGATGAC	160
Peroxisome proliferator-activated receptor gamma 2 (<i>Pparg</i>)	GCGATTCCTTCACTGATACTG' GAGTGGGAGTGGTCTTCCATTAC	136
Lipoprotein lipase (<i>lpl</i>)	GGCGTACCTTGAGATAGAGTTCTG TGTTTTCTACAGGGTGCTTTAGATGAC	113
Fatty acid-binding protein (<i>FABP-4</i>)	CCAGGAATTTGACGAAGT TCTCTTTATGGTGGTTGATT	118
Heme oxygenase-1 (<i>OH-1</i>)	CTATCGTGCTCGCATGAAC CAGCTCCTCAAACAGCTAA	118
Glyceraldehyde 3-phosphate dehydrogenase (<i>GAPDH</i>)	ACTCCATTCTCCACCTTT TTACTCCTTGAGGCCATGT	143

4°C overnight with antibodies specific for HO-1(1:500) (Stressgen Biotechnologies, Victoria, Canada) and mouse anti- β -actin (1:500, polyclonal; Zhongshan Gold Bridge, Beijing, China) followed by secondary antibodies (1:3,000) in PBS-Tween-20. The proteins were visualized *via* enhanced chemiluminescence (Millipore Corp., Molsheim, France) followed by exposure to X-ray film (Kodak, Beijing, China). For quantification, the intensities of the bands were assessed with Image-Pro plus 6.0 (Rockville, MD, USA) and are expressed as optical density units relative to β -actin. Results were presented as mean \pm SD for 3 separate experiments, with OH-1 expression levels to that of β -actin.

Statistical Analysis

The results are presented as the means \pm standard deviations and were analyzed in SPSS software (version 17.0 for Windows Smart Viewer; SPSS Inc., Chicago, IL, USA) with one-way analyses of variance or Student's *t*-tests as appropriate. A *p*-value of <0.05 was regarded as statistically significant.

Results

Cell Viability Characteristics of Surface Phenotypes, Morphology, Growth, and Differentiation of Isolated Rat BMSCs

Cells viability (MTT assay) was significantly affected by lycopene treatment at 7 and 14 days of study. A significant decrease in cells viability was evident at 15- and 20-mM lycopene by 92.3 ± 5.6 and 80.1 ± 8.5 % (at 7 days) and by 86.5 ± 10.4 and 78.6 ± 9.4 % (at 14 days) of control ($p < 0.05$); respectively (Figure 1).

At passage 2, 75.12% of the cells were positive for CD44 and >95.16 % were positive for CD90, whereas 4.11% and 0.51% were negative for CD45 and CD11b, respectively, by flow cytometry (Figure 2A). These results demonstrated that most of the cells expressed the BMSC surface markers and thus were used for the various studies. The cellular growth curve showed a lag phase appearing after approximately 24-48 h of culture, which was followed by logarithmic growth and then growth arrest. The mean doubling time of the cultured cells were approximately 35 h. Roughly 85-92% of cells were in the G_0 - G_1 phase, confirming that most of the rat BMSCs in culture

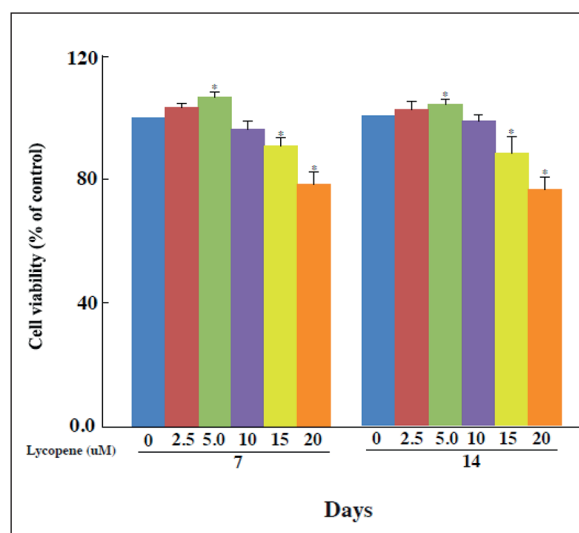


Figure 1. Cell viability. Cell viability was determined by using MTT assay. Cells were incubated for 7, 10 and 14 days and heated with 1.25-25 μ M lycopene. Results are shown for 7 and 14 days, and data were presented as mean \pm SD ($n=3$). * $p < 0.05$ vs. control (without lycopene).

were quiescent, with only a small percentage of actively proliferating cells.

Effect of Lycopene on ALP Activity in Rat BMSCs

High levels of ALP activity are indicative of an osteogenic phenotype. As expected, cells cultured in OM exhibited time-dependent ALP stimulation with activities that were markedly higher than those in cells cultured in CM for 6 days (Figure 2B). The activity of cells in OM was 1.5-fold that of cells cultured in CM after 12 days. Treatment with lycopene enhanced ALP activity, with the greatest increase observed at 7 and 14 days. Rat BMSCs consistently showed dose-dependent increases in ALP activity in response to lycopene treatment (Figure 2B).

Effect of Lycopene on Osteocalcin Secretion in Cultures of Rat BMSCs

Osteocalcin (a marker of bone formation) is a non-collagen protein released by osteoblasts. During the early stages of osteogenic culturing of r-BMSCs (0-3 days), no evident change was observed in response to lycopene treatment. Osteocalcin levels were significantly higher in response to lycopene treatment in cells cultured over 6-9 days (an increase by 1.9-2.3-fold) as compared with respective controls (* $p < 0.05$, ** $p < 0.01$). Beyond 6-9 days of cell culture, os-

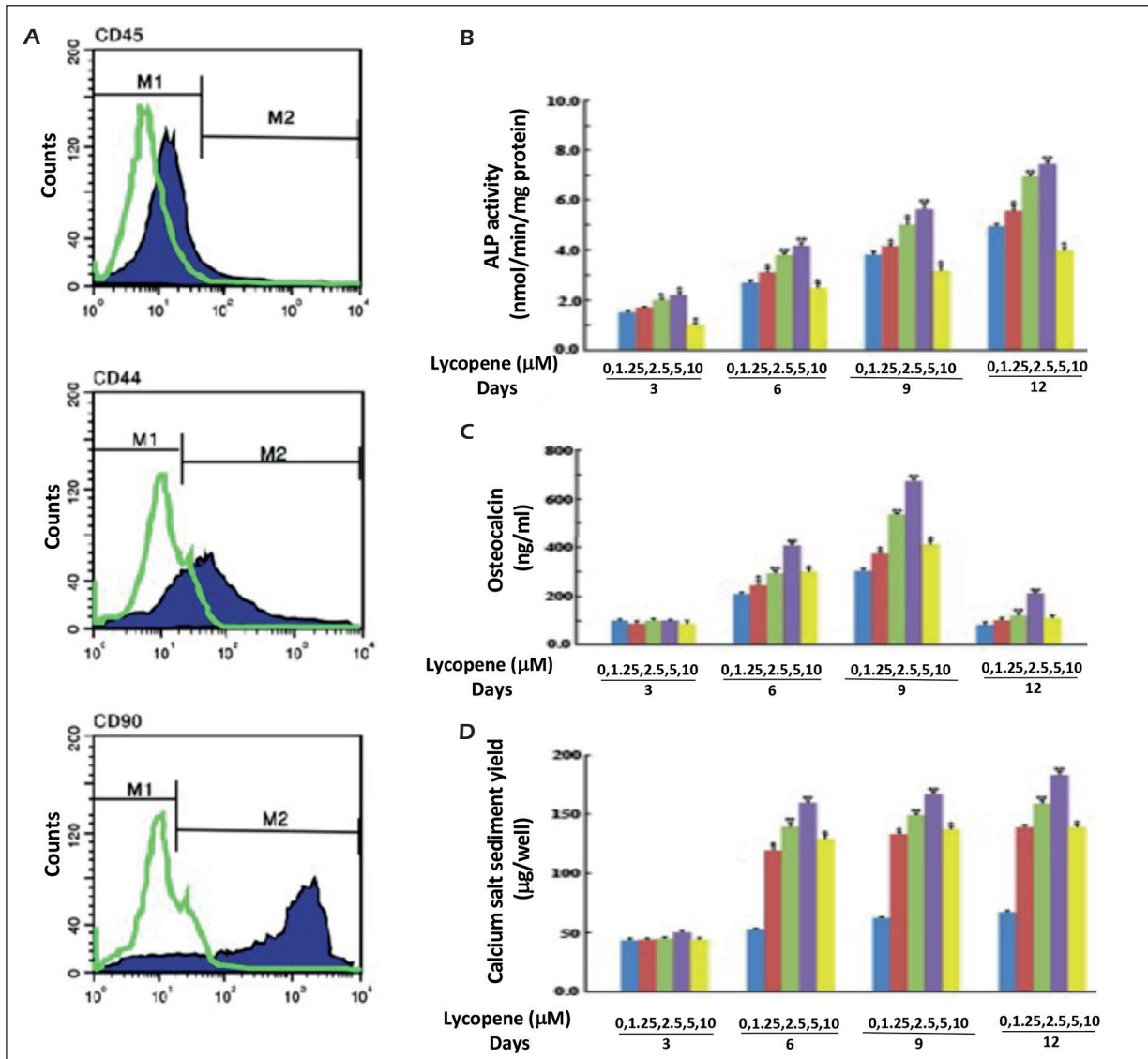


Figure 2. A, Purity and phenotype characteristics of rat bone marrow mesenchymal stem cells (rBMSCs), B, The effect of lycopene on stimulating osteogenic differentiation of rBMSCs as indicated by ALP/activity (nmol/min/mg protein). C, The effect of lycopene on stimulating the release of osteocalcin (ng/ml) during osteogenic differentiation of rBMSCs. D, The effect of lycopene on calcium deposition (ug/well) during osteogenic differentiation of rBMSCs. For (A), cells were incubated in the presence of fluorochrome – labelled primary antibodies against CD₄₄, CD₄₅, CD₉₀ or CD_{11b} and the corresponding isotype control. For stained cells were then examined by flowcytometry. For (B), the activity of ALP was determined (the absorbance at 570nm – mean ± 6 replicate/cultures) during 3-12 days of cell cultures. For (C), the released osteocalcin into culture (measured by rat – specific EIA osteocalcin kit – mean ± 6 replicate cultures) during 12 days of cell cultures. For (D), the amount of calcium salt sediment produced (determined by O-cresolphthalein – complex method with colorimetric array kit – mean ± 6 replicate cultures) during 12 days of cell cultures. **p*<0.05, ***p*<0.01 vs. control (without lycopene in the culture medium).

teocalcin secretion declined and differences between lycopene treatment and the corresponding control disappeared, yet the cells treated with lycopene produced more osteocalcin compared with controls [2.5-5.0 mM, *p* <0.05, ***p* <0.01 vs. control (without lycopene in the culture medium)] (Figure 2C).

Effect of Lycopene on Matrix Mineralization in Cultures of Rat BMSCs

Rat BMSCs cultured in medium with or without lycopene were examined after Alizarin red-S staining. Mineralization was observed at 5.0 mM lycopene (an increase by about 2.8- and 2.4- fold, **p*<0.001) for those cultured for 9 and 12 days

in OM (Figure 2D) respectively, however, cells cultured in the presence of 1.25 or 2.50 mM lycopene exhibited 2.0-fold and 2.5-fold amounts of matrix mineralization (for the same time interval), respectively, compared with those in CM (Figure 2D). Thus, the calcium deposition results confirm the findings performed by staining of Alizarin Red S Analysis.

Effect of Lycopene on Osteoblastogenesis

Cells cultured in OM supplemented with lycopene exhibited alterations in gene expression and in the osteoblast phenotype. During differentiation, the expression of genes increased relative to that from cells cultured in OM without lycopene (Figure 3). In particular, the expression of the transcription factor *Runx2*, which, along with *Sp7*, is crucial for osteoblast differentiation and bone formation, was markedly higher after 7 days in culture. A high lycopene concentration (5 mM) resulted in the highest expression of *Runx2* and *Sp7* (Figure 3A, 3B). The presence of lycopene (particularly at 5 mM) in the OM also produced a significantly higher *Alpl* expression (Figure 3C). Furthermore, lycopene treatment also increased the transcription of genes involved in cellular matrix formation in cells cultured in OM. After 7 and 14 days of supplementation, the expression of *Coll1a1* was increased with 2.5- and 5-mM lycopene, and the expression of *Bglap* was highest after 14 days of treatment with 5 mM lycopene (Figure 3D, 3E). Finally, lycopene supplementation to cells cultured in OM increased the *Tnfrsf11b/Tnfsf11* ratio compared to that in cells cultured without lycopene (Figure 4).

Effect of Lycopene on Adipogenesis

The expression of three genes that are markers for adipogenesis, *Pparg*, *Lpl*, and *Fabp4*, were analyzed (Figure 3F, 3G, 3H). The expression of *Pparg* was inhibited by lycopene treatment at all time intervals and all concentrations studied (Figure 3F). Maximum inhibition was obtained at 14 days. In addition, the expression of *Lpl* was most strongly inhibited after 14 days of differentiation in the presence of lycopene (Figure 3G). Furthermore, *Fabp4* transcription was inhibited in cells cultured in AM in the presence of lycopene (Figure 3H).

The effect of lycopene supplementation on the adipocyte phenotype was also studied. For this, lipid droplet formation, a feature specific

to adipocyte formation, was examined with Red Oil O staining. Drastic phenotype changes were observed in differentiating rat BMSCs treated with lycopene, demonstrated by decreased development and an accumulation of cellular fat. Additionally, lycopene treatment decreased the area occupied by lipid droplets compared with that from cells cultured in AM (results not shown) and corresponding cultures in CM.

Lycopene Increases the Heme Oxygenase (HO)-1 Expression

To examine how lycopene affects rat BMSC differentiation, the expression of HO-1 was studied. Lycopene treatment of r-BMSCs cultured for 12 h in OM, increased HO-1 protein expression in a dose-dependent manner (Figure 5A, 5B). In addition, lycopene at 5.0 mM induced a marked increase in protein expression of HO-1 for the time intervals examined with a maximum response of 2.1 ± 0.38 -fold increase following a 12 h treatment (Figure 5B). Moreover, lycopene treatment significantly increased the expression of HO-1 at the mRNA levels in rat BMSCs cultured for 14 days in OM (Figure 5C). Thus, lycopene appears to enhance osteoblast differentiation *via* the upregulation of HO-1.

Discussion

Carotenoids are important among dietary antioxidants mainly found in fruits and vegetables. Among the main carotenoids consumed by human, lycopene is very important, and is considered to exhibit the most potent antioxidative capacity-being twice and ten times that of β -carotene and α -tocopherol, respectively^{16,17}. Some human studies^{34,35} demonstrated positive relationships between the fruit intake and the bone mineralization density (BMD) values. Furthermore, due to its potent antioxidative effects, high plasma levels of lycopene correlated with a decrease in the risk of various types of cancer, including prostatic, cervical, pancreatic and gastrointestinal, as well as in patients with myocardial infarction^{16,17}. Also, several animals and human studies^{19,21} demonstrated the protective effects of lycopene to bone health. Collectively, these observations demonstrated the crucial need to provide more information on the molecular effects of lycopene at the cellular level to understand the protective effects of lycopene on bone health.

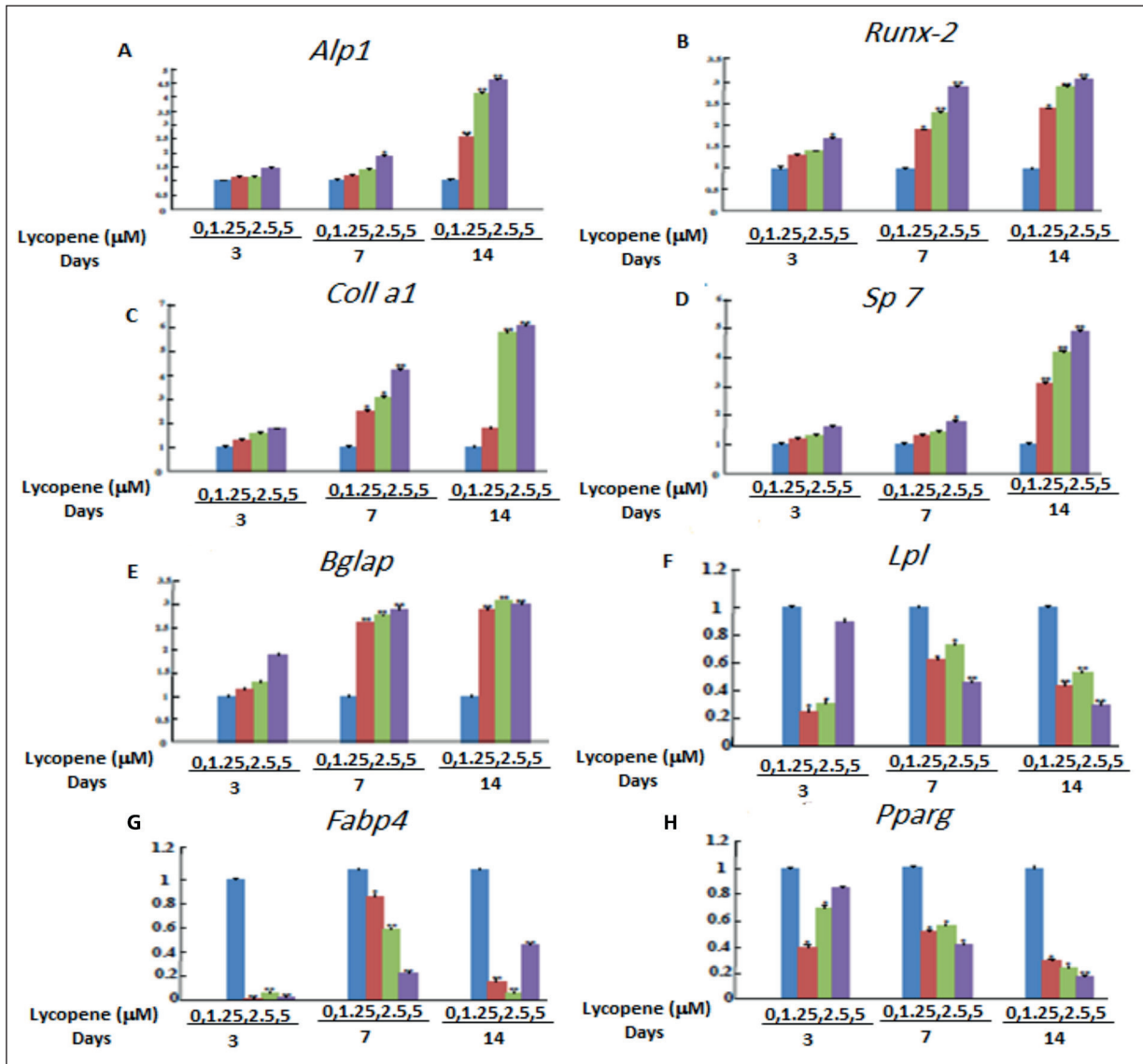


Figure 3. qCT-PCR analysis of genes involved in osteoblastogenesis and adipogenesis media in the presence of various concentrations of lycopene (0, 1.5, 2.5, 5.0 and 10.0 μM) over different days (3-14 days). The analysis of the expression of genes involved in osteoplastogenesis as evidenced by the transcription of (A) Alkaline phosphatase (*Alpl*), (B) runt – related transcription factor 2 (*Runx2*), (C) collagen type I, alpha 1 (*Colla1*), (D) Osterix (*Sp7*) and (E) osteocalcin (*Bglap*). The expression of genes involved in adipogenesis as evidenced by the transcription of (F) lipoprotein lipase (*Lpl*), (G) fatty acid-binding protein-4 (*Fabp4*) and (H) peroxisome proliferator -activator receptor gama-2 (*Pparg*) and glyceraldehyde-phosphate dehydrogenase (*Gapdh*). Results are expressed as mean ± SD fold increase relative to the non-supplemented control (without lycopene) (for 3 culture assays at each time interval).

The results of this study demonstrated that, lycopene affects the differentiation of rat BM-SCs into osteoblasts or adipocytes. Lycopene enhanced osteoblastogenesis *in vitro*, as indicated by an increased transcription of osteogenic genes and an increase in phenotypic osteoblast characteristics, resulting in extracellular matrix mineralization. Specifically, the expression of both master osteoblastic transcription factors,

Runx2 and *Sp7*, sequential factors, that are key in inducing osteogenic differentiation, was increased. Accordingly, *Runx2* deficiency results in an arrest of osteoblast maturation and a lack of bone formation with concomitant differentiation of adipocytes³⁶. Likewise, BMSCs from *Sp7*-null mice failed to differentiate into osteoblasts, resulting in no bone formation³⁶. Thus, both *Runx2* and *Sp7* participate in the cellular commitment

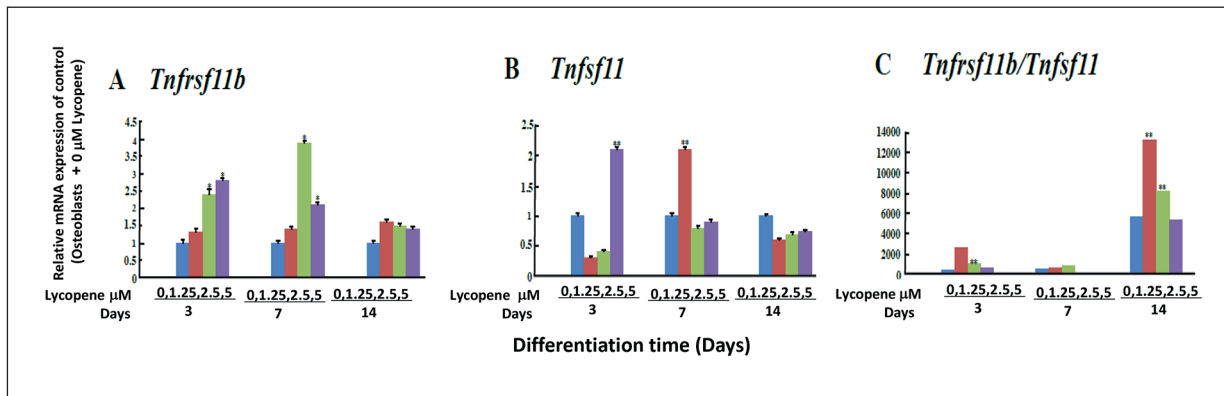


Figure 4. Time course changes in mRNA expression of (A) Osteoprotegrin (*Tnfrsf11b*) and (B) Receptor activator for nuclear factor KB ligand (*Tnfsf11*) and (C) both *Tnfrsf11b*/*Tnfsf11* in rBMSCs cultured for 3-14 days. Results are expressed as mean \pm SD fold increase relative to the non-supplemented control (without lycopene) (n= 3 culture assays at each time interval). * p <0.05; ** p <0.01 vs. control.

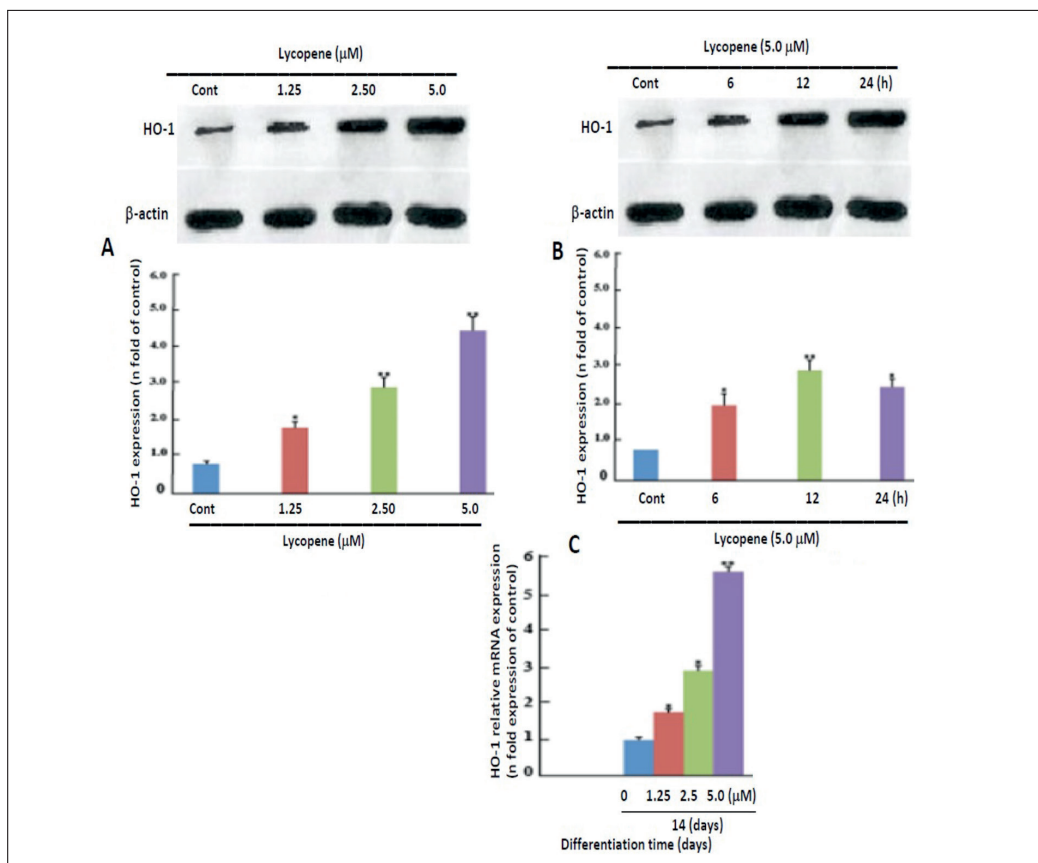


Figure 5. Lycopene supplementation increases the expression of hydroxylase – 1 (OH-1). The (C) rBMSCs were cultured for 14 days in osteogenic media in the presence (5 μ M lycopene) or absence (control), respectively. A, Rat BMSCs were cultured for 12h as indicated. B, Cells were incubated with lycopene for the time intervals shown. The OH-1 protein expression was analyzed using western blot. Results are expressed as means \pm SD for 6 replicates, * p <0.001 vs. control. C, The total RNA was isolated and analyzed by real-time PCR. Results are expressed as mean \pm SD fold increase relative to the non-supplemented control (without lycopene) (n= 6 culture assay). * p <0.001 vs. control.

to osteoblast differentiation and the maintenance of osteoblast-specific expression during osteoblastogenesis³⁶. The upregulation of *Runx2* and *Sp7* observed in the present study indicates that lycopene dose-dependently enhances both early and later phases of osteoblast differentiation. This effect was likely facilitated by the induced transcription of *Alpl* and *Colla1*, considered early and mid-phase markers of BMSC differentiation and matrix synthesis, and *Bglap*, which influences later stages of bone formation and is also closely related to osteoblastic maturation and matrix mineralization^{37,38}. These genes, expressed sequentially, contribute to extracellular matrix mineralisation³⁹. Accordingly, their upregulation in the rat BMSCs supported matrix synthesis and maturation and led to larger amounts of calcium deposits. Consistent with these observations, increases in ALP activity and osteocalcin secretion were observed. Furthermore, Ardawi et al²¹ reported that *Colla1* expression was increased in individuals who ingested lycopene.

Due to the regulatory actions of *Tnfsf11* and *Tnfrsf11b* on osteoclasts and other bone cells, the *Tnfsf11/Tnfrsf11b* ratio is indicative of the bone resorption rate⁴⁰. In our study, an increase in this ratio was observed in cells treated with lycopene, which suggests that lycopene negatively impacts bone resorption. Osteoprotegrin (encoded by *Tnfrsf11b*) is expressed by osteoblasts and differentiating BMSCs and binds the ligands of receptor activator for nuclear factor κ B (encoded by *Tnfsf11*) on osteoblasts, thereby inhibiting their differentiation⁴⁰ and diminishing the activity and promoting the apoptosis of remaining osteoclasts. Hence, whereas high levels of osteoprotegrin block the loss of bone by lowering bone resorption may promote increased bone mineral density and skeletal strength, its deficiency promotes osteoporosis^{40,41}. In addition, extracellular matrix mineralization *in vitro* was enhanced by lycopene (1.25-5.00 mM). Our results are consistent with those from the few studies on the effects of lycopene on osteoblastogenesis from human BMSCs¹⁹ and human osteoblast-like osteosarcoma SaOS-2 cells⁴². Moreover, lycopene (>20 mM) increased the viability of mouse MSCs under hypoxia, with no effect when cells were under normoxia⁴³.

Peroxisome proliferator-activator receptor gamma 2 (PPAR γ 2) is a nuclear transcription factor that directs MSCs to differentiate into adipocytes by regulating the transcription of adipogenic genes⁴⁴. The PPAR γ 2 is activated by several ligands that are endogenous products of

oxidative stress⁴⁵ and exhibits pro-adipocytic and anti-osteoblastic effects⁴⁴; its inhibition suppresses adipogenesis⁴⁶. For the first time, we showed that adipogenic differentiation of BMSCs is significantly inhibited by lycopene, which inhibited the transcription of *Pparg*. PPAR γ 2 is reported to indirectly inhibit osteoblastic differentiation via suppressing *Runx2* transcription⁴⁷, which is consistent with the findings of the present study. Accordingly, the transcription of other genetic markers of adipocytes, namely, *Lpl* and *Fabp4*⁴⁸, was suppressed along with a reduction in fat accumulation. Thus, the results of our study indicate that lycopene treatment promotes osteoblastic differentiation at the expense of adipocyte differentiation in part by downregulating *Pparg* and antagonising the possible effects of endogenous oxidised and/or exogenous ligands of PPAR γ 2.

The imbalance in bone marrow cellular differentiation between osteoblastogenesis and adipogenesis is concurrent with an increase in osteoclastogenesis, representing a key mechanism in pathological conditions such as osteoporosis, diabetes, and aging⁴⁹. Indeed, several studies⁵⁰⁻⁵² demonstrated that the ovariectomized rats and patients with osteoporosis, exhibited the accumulation of bone fat was concurrent with bone loss. Patients with osteoporosis exhibit higher levels of oxidative stress, and cumulative oxidative stress impairs antioxidative defense systems, causing an imbalance of the redox mechanism among the elderly⁵³. Thus, oxidative stress might explain the molecular changes that link osteoporosis and aging or age-related pathologies.

Oxidative stress is a major factor impairing MSC function, reducing osteogenesis and favoring adipogenesis. HO-1 is known to attenuate the overall production of ROS through its antioxidative activities in the bone microenvironment⁶¹, demonstrating a key role of ROS balance in osteogenic differentiation. HO-1 is also implicated in BMSC differentiation and modulates osteoblastogenesis and osteoclastogenesis⁵⁴ via diverse signaling pathways^{55,56}. HO-1 also regulates the generation of osteoblasts during bone repair⁵⁷ and enhances osteoblast differentiation from MSCs⁵⁸, indicative of roles in bone development and osteogenesis. Furthermore, HO-1 may regulate fat metabolism and adipogenesis, as an upregulation of HO-1 was found to suppress adipocyte differentiation⁵⁹, an effect that was also observed when overexpressed in porcine cells⁵⁵. The HO-1 induction *in vitro* elicited a significant inhibitory effect on osteo-

clastogenesis⁶⁰. Most recently, Liu et al⁶¹ found that HO-1 potentiates osteogenic differentiation and inhibits adipogenic differentiation induced by bone morphogenetic protein 9 in murine pluripotent cells *via* multiple signaling pathways. Thus, the expression of HO-1 coupled with a decrease in ROS, are considered essential for osteoblast differentiation of MSCs. In our study, we provided new evidence that lycopene treatment significantly increased the expression of HO-1 at the mRNA levels. In addition, the HO-1 protein expression can be induced with lycopene in a dose-dependent manner in r-BMSCs. Lycopene also induces the transcription of genes implicated in osteogenesis, such as those encoding superoxide dismutase, glutathione peroxidase, and catalase²¹. These observations support our findings of the role of HO-1 in the differentiation of osteoblasts from rat BMSCs and suggest a mechanism by which lycopene exerts these effects. Lycopene can activate MAP kinase in tumor cells to induce the expression of HO-1⁶². This key signal transduction pathway might regulate the differentiation of MSCs into osteoblasts, as several growth factors that activate MAP kinase can also inhibit adipocyte differentiation. Furthermore, the activation of MAP kinase by leptin suppresses adipogenic differentiation by PPAR γ phosphorylation⁶³. More recently, Costa-Rodrigues et al¹⁸ showed direct effects of lycopene on osteoclastogenesis *via* MAP kinase and PKC pathways and on osteoblastogenesis *via* MAP kinase and nuclear factor κ B signaling. Such findings complement those of the present study, though more studies are still needed.

Conclusions

In summary, the findings presented in our study suggested that the bone loss due to aging and other pathological conditions may be prevented from lycopene directly by inducing gene transcription during early and middle stages of osteogenesis, resulting in the differentiation of MSCs into osteoblasts and the inhibition of adipogenesis, and indirectly by modulating osteoclast differentiation *via* regulating the *Tnfrsf11/Tnfrsf11b* ratio. Other important effects of lycopene on the cellular processes may include the stimulation of gap junction communication and production of detoxifying enzymes. Thus, maintaining a dietary intake of lycopene may

support bone structure, strength, and function²¹. The effects of lycopene have potential relevant clinical implications, as nano formulations of lycopene could be used as a dietary supplement or encapsulated as a drug^{21,30}. This represents an attractive therapeutic approach due to the very limited side effects compared with those of the known osteoporosis medications, of which some have serious adverse effects or are unaffordable⁶⁴. Further studies are needed to confirm these observations in human MSCs and *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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