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ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Dr. Mohammed Grawish is a Distinguished Professor at Mansoura University and Vice-Dean for Community Services and Environmental Affairs at Delta University for Science and Technology (Egypt). Dr. Grawish received his Bachelor's degree (1990), Master's degree in Oral Biology (1998), and his PhD (2003) from the Faculty of Dentistry, Mansoura University. After, he worked as Lecturer in the Al-Gabl Al-Garby University (2005-2008; Gehrian, Libya) and as Associate Professor in the King Saud University (2011-2013; Riyadh, Saudi Arabia). His ongoing research interests focus mainly on the appropriate therapeutic use of stem cells in dentistry, the design and characterization of biomaterials as scaffold materials for loading stem cells, and the application of complementary and alternative medicine as an adjunctive treatment to traditional medicine for oral diseases. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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ORIGINAL ARTICLE

Basic Study

Effects of normobaric cyclic hypoxia exposure on mesenchymal stem-cell differentiation–pilot study on bone parameters in elderly

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Abstract

BACKGROUND

Mesenchymal stem cells (MSC) of bone marrow are the progenitor of osteoblasts and adipocytes. MSC tend to differentiate into adipocytes, instead of osteoblasts, with aging. This favors the loss of bone mass and development of osteoporosis. Hypoxia induces hypoxia inducible factor 1α gene encoding transcription factor, which regulates the expression of genes related to energy metabolism and angiogenesis. That allows a better adaptation to low O_2 conditions. Sustained hypoxia has negative effects on bone metabolism, favoring bone resorption. Yet, surprisingly, cyclic hypoxia (CH), short times of hypoxia followed by long times in normoxia, can modulate MSC differentiation and improve bone health in aging.

AIM

To evaluate the CH effect on MSC differentiation, and whether it improves bone mineral density in elderly.

METHODS

MSC cultures were induced to differentiate into osteoblasts or adipocytes, in CH (3% O_2 for 1, 2 or 4 h, 4 d a week). Extracellular-matrix mineralization and lipid-

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droplet formation were studied in MSC induced to differentiate into osteoblast or adipocytes, respectively. In addition, gene expression of marker genes, for osteogenesis or adipogenesis, have been quantified by quantitative real time polymerase chain reaction. The *in vivo* studies with elderly (> 75 years old; $n = 10$) were carried out in a hypoxia chamber, simulating an altitude of 2500 m above sea level, or in normoxia, for 18 wk (36 CH sessions of 16 min each). Percentages of fat mass and bone mineral density from whole body, trunk and right proximal femur (femoral, femoral neck and trochanter) were assessed, using dual-energy X-ray absorptiometry.

RESULTS

CH (4 h of hypoxic exposure) inhibited extracellular matrix mineralization and lipid-droplet formation in MSC induced to differentiate into osteoblasts or adipocytes, respectively. However, both parameters were not significantly affected by the other shorter hypoxia times assessed. The longest periods of hypoxia downregulated the expression of genes related to extracellular matrix formation, in MSC induced to differentiate into osteoblasts. Interestingly, osteocalcin (associated to energy metabolism) was upregulated. Vascular endothelial growth factor an expression and low-density lipoprotein receptor related protein 5/6/dickkopf Wnt signaling pathway inhibitor 1 (associated to Wnt/β-catenin pathway activation) increased in osteoblasts. Yet, they decreased in adipocytes after CH treatments, mainly with the longest hypoxia times. However, the same CH treatments increased the osteoprotegerin/receptor activator for nuclear factor kappa B ligand ratio in both cell types. An increase in total bone mineral density was observed in elderly people exposed to CH, but not in specific regions. The percentage of fat did not vary between groups.

CONCLUSION

CH may have positive effects on bone health in the elderly, due to its possible inhibitory effect on bone resorption, by increasing the osteoprotegerin / receptor activator for nuclear factor kappa B ligand ratio.

Key Words: Normobaric cyclic hypoxia; Mesenchymal stem cells; Cellular differentiation; Osteoblasts; Adipocytes; Bone health

Core Tip: Cyclic hypoxia (CH) can improve bone health in aging. Mesenchymal stem cells were induced to differentiate into osteoblasts or adipocytes, in hypoxia (3% O_2) for 1, 2 or 4 h, 4 d a week. Older people were treated with 36 CH sessions of 16 min (simulating an altitude of 2500 m), for 18 wk. Exposure to longer periods of hypoxia inhibited mesenchymal stem cells differentiation, but increased osteoprotegerin/receptor activator for nuclear factor kappa B ligand expression in both cell types. CH increased total bone mineral density, but not fat content, in elderly. CH may improve bone health in elderly, due to its inhibitory effect on bone resorption, by increasing the osteoprotegerin/receptor activator for nuclear factor kappa B ligand ratio.

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INTRODUCTION

The rates of diseases and conditions affecting older individuals have noticeably increased since the Age of Enlightenment, due to an extension in human longevity[1].

Old age is associated, among other factors, with a decline in bone mass and strength. It can culminate with osteoporosis, which provokes significant morbidity and mortality, increasing healthcare costs worldwide[2]. In this sense, accumulating evidence indicates a complex relationship between adiposity and osteoporosis[3]. Osteoblasts and adipocytes originate from the common-precursor mesenchymal stem cells (MSC). The balance between differentiation of osteoblasts and adipocytes is regulated by both intra- and extra-cellular factors[1]. Aging alone alters the fate of MSC in bone marrow, by promoting adipogenesis and reducing osteoblastogenesis[4], increasing age-related bone loss[5].

Also, proinflammatory cytokines associated with obesity and aging regulate receptor activator for nuclear factor kappa B ligand/receptor activator for nuclear factor kappa B/osteoprotegerin (RANKL/RANK/OPG) pathways, stimulating osteoclast activity and bone loss[6]. Osteoclastogenesis is induced in osteoclast precursor cells when RANKL, produced by osteocytes and osteoblasts, binds to its RANK receptor. However, osteoblasts also express OPG, which encodes a RANK decoy, and thus inhibits RANKL binding and activation of osteoclastogenesis. Therefore, the relationship between the expression of *OPG* and *RANKL* genes regulates bone resorption[7]. Interestingly, oxygen variations have also been established as another factor that could influence tissue remodeling[8]. Bone marrow is maintained in a hypoxic state in the body[9]. Besides, various genes regulated by the hypoxia inducible factor 1α (HIF-1A) mediate different effects[10].

Although little is known about the metabolic pathways used by bone cells, the glucose metabolism may play a prominent role during osteoblast differentiation[11]. Even in the presence of oxygen, bone cells metabolize glucose by a phenomenon known as the Warburg effect or aerobic glycolysis[10]. On the other hand, HIF-1A regulates bone remodeling, involving genes as vascular endothelial growth factor, erythropoietin and OPG[12]. The former represents a key player in coupling angiogenesis and osteogenesis[10], as well as promoting osteogenesis[13]. On the other hand, erythropoietin has also been shown to stimulate bone formation and repair. Similarly, to this mechanism of physiological hypoxic, some evidence has shown the relevance of inhalation of air with reduced oxygen content. Thus, hypoxia is a nonpharmacological method used as prevention and treatment strategy in many clinical conditions[8,14]. However, the pattern (intermittent or chronic) and the severity (duration and intensity) of hypoxia episodes are key determinants of its physiological impact[13,15,16].

Many *in vitro* experiments have studied the effects of hypoxia on proliferation and differentiation of human MSC, along adipogenic and osteogenic lineages, with varying results[17-19]. Adipogenic and osteogenic differentiation were severely impaired under severe hypoxia, but increasing oxygen levels from 1% to 3% restored the osteogenic differentiation capacity of cells[9,20]. Furthermore, hypoxia regulates the differentiation of MSC along osteogenic lineages in a time-dependent manner, reducing it in continuous hypoxic conditions[20]. According to aforementioned studies, *in vivo* research on animal and human development suggests that chronic severe hypoxia[21-23] may lead to deleterious consequences on bone system. Likewise, moderate intermittent hypoxia may lead to positive adaptations[13,24]. However, there is currently an imprecise terminology about the various types of intermittent hypoxia exposure[25]. That is usually associated with sleep-disordered breathing. It is different from cyclic hypoxia (CH), defined as periodic exposure to hypoxia, from a few minutes to days. That is repeated over a number of days, being interrupted by normoxic exposure, or lower levels of hypoxia[26].

Many cycles of hypoxia and re-oxygenation activate HIF-1A during intermittent exposure. But HIF-1A-strictly accumulates during periods of hypoxia, in cyclic exposure. That represents an essential aspect of the beneficial effects of cyclic hypoxia[25]. Thus, in contrast to severe of intermittent hypoxia (*e.g.*, cells cultures under < 2% O_2 or 3%-8% inspired O_2), moderate CH protocols (cells cultures under 2%-9% O_2 or 9%-16% inspired O_2) may reinforce the immune system, while suppressing the production of pro-inflammatory mediators. Therefore, CH holds potential for bone remodeling applications. Yet, more research is required to elucidate the mechanisms responsible for such possible adaptations. They should allow to define optimal protocols and practical guidelines.

Therefore, the aim of this study was to investigate the effects of CH exposure on differentiation of human bone-marrow derived MSC into osteoblasts or adipocytes. In addition, we performed a pilot study, in elderly, on how CH exposure may affect bone mineral density (BMD) and fat mass.

MATERIALS AND METHODS

Mesenchymal stem cells culture and differentiation

Cryopreserved MSC from our research group's collection were used. They were from bone marrow of donors, between 20 and 35 years old, recruited through the Hematology service, under the Bone Marrow Transplantation Program, at the Reina Sofía University Hospital (Córdoba, Spain)[27]. Cultures were grown as we have previously reported[27]. In short, MSC were thawed and seeded in 75 cm^2 flasks from Nalgene-Nunc-Thermo Fisher Scientific (Waltham, MA, United States). They were grown in Minimum Essential-Medium Alpha from Cambrex Bio Science-Lonza (Basel, Switzerland), containing 2 mmol/L UltraGlutamine (Lonza), 15% fetal bovine-serum (Gibco-Thermo Fisher Scientific), 100 U ampicillin, 0.1 mg streptomycin/mL and 1 ng basic fibroblast-growth factor (bFGF)/mL from Sigma-Aldrich (Saint Louis, MO, United States). Cultures were incubated at 37 ºC with 95% humidity and 5% CO_2.

The medium was changed each three to four days. Cells were detached with trypsin/EDTA (Lonza), when reaching near 90% confluence. Then, they were seeded in culture plates with the same medium, but with 10% fetal bovine-serum, at a density of about 1000 cells/cm^2. When cultures were near confluence, they were induced to differentiate into osteoblasts or adipocytes. Osteoblastic differentiation was induced with 10^{-8} mol/L dexamethasone, 0.2 mmol/L ascorbic acid and 10 mmol/L β-glycerolphosphate. On the other hand, adipogenic differentiation was triggered with 5 × 10^{-7} mol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine and 50 μM indomethacin (all inducers were from Sigma-Aldrich). Adipocyte and osteoblast differentiation were maintained for 14 and 21 d, respectively. Cultures were maintained in normoxia or cyclic hypoxia during differentiation (3% O_2). The exposure was for 1, 2 or 4 h in hypoxia per day, four days per week, distributed as indicated in Figure 1.

Mineralized extracellular-matrix (osteoblasts) and lipid droplets (adipocytes) staining

Alizarin-red staining at day 21 of osteoblastic differentiation was used for extracellular-matrix mineralization quantification. In short, cultures in P12 plates were fixed with 3.7% formaldehyde for 10 min. Then, they were stained with 40 mmol/L of alizarin red in water, adjusting pH 4.15 with ammonium hydroxide (both chemicals from Sigma-Aldrich) for 10 min. Wells were then washed several times with 60% of isopropanol, dried and visualized under light microscopy. Alizarin-red deposit measurements were carried out after elution with 10% acetic acid, neutralization with 10% ammonium hydroxide and 405 nm spectrophotometric-absorbance quantification.

On the other hand, lipid-droplets accumulation was determined in cultures by oil-red O staining, at day 14 after starting differentiation into adipocytes. Briefly, cultures in P12 plates were fixed with 3.7% formaldehyde for 20 min, washed with distilled water and stained with a solution containing 60% of 0.35% oil red (w/v in isopropanol) and 40% of distilled water, for 15 to 20 min. Then, wells were washed with water, stained with hematoxylin and visualized with optical microscope. Oil-red O staining was carried out eluting it with isopropanol at room temperature, for 10 min. The spectrophotometric-absorbance of eluates was checked at 510 nm. Values were normalized, after estimating the number of cells per well, by crystal-violet staining. In short, after oil red elution, cells were stained with 0.1% crystal violet in 10% ethanol, for 20 min. Then, they were washed with distilled water, and the stain was eluted with 10% acetic acid for 20 min. Absorbance at 590 nm was measured in the resulting eluate. Lipid accumulation in cultures was expressed as absorbance ratio (A510 nm/A590 nm).

RNA isolation and quantitative real time polymerase chain reaction

RNA from the different culture cells was isolated using the NZY total RNA isolation kit from NZYTech (Lisbon, Portugal), following the manufacturer' directions. RNA was quantified with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific), and 900 ng were retrotranscribed with iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA, United States).

Quantitative real time polymerase chain reaction was carried out in a LightCycler 480 Instrument from Roche Applied Science (Penzberg, Germany). Each reaction contained one μl of cDNA, 10 pmol of each primer pair (Table 1) and SensiFAST Sybr No-Rox Mix from Bioline (London, United Kingdom). The polymerase chain reaction amplification profile included one cycle at 95 °C for 2 min (DNA denaturation and hot-start DNA-polymerase activation) and 45 amplification cycles: 94 °C for 5 s (DNA

Table 1 Primer sequences and product sizes

Gene	Primer sequence (5' —> 3')	Product size (bp)
Runt-related transcription factor 2 (*RUNX2*)	TGGTTAATCTCCGCAGGTCAC; ACTGTGCTGAAGAGGCTGTTTG	143
Osterix (*SP7*)	AGCCAGAAGCTGTGAAACCTC; AGCTGCAAGCTCTCCATAACC	163
Collagen, type I, alpha 1 (*COL1A1*)	CGCTGGCCCCAAAGGATCTCCTG; GGGGTCCGGGAACACCTCGCTC	263
Integrin-binding sialoprotein (*IBSP*)	AGGGCAGTAGTGACTCATCCG; CGTCCTCTCCATAGCCCAGTGTTG	171
Osteocalcin (*BGLAP*)	CCATGAGAGCCCTCACACTCC; GGTCAGCCAACTCGTCACAGTC	258
Vascular endothelial growth factor A (*VEGFA*)	GCGAGCAGCGAAAGCGACAGG; CGTCAGCGCGACTGGTC	114
Peroxisome proliferator-activated receptor gamma 2 (*PPARG2*)	GCGATTCCTTCACTGATACACTG; GAGTGGGAGTGGTCTTCCATTAC	136
Fatty acid synthase (*FASN*)	AAGCTGAAGGACCTGTCTAGG; CGGAGTGAATCTGGGTTGATG	146
Adipose triglyceride lipase (*ATGL*) or patatin-like phospholipase domain-containing protein 2 (*PNPLA2*)	CCAACACCAGCATCCAGTTCA; ATCCCTGCTTGCACATCTCTC	102
Glycerol-3-Phosphate Dehydrogenase 1 (*GPD1*)	ATACAGCATCCTCCAGCACAAG; GGATGATTCTGCAGGCAGTG	120
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1A*)	CTGGTTGCCTGCATGAGTGTG; GGGCAAAGAGGCTGGTCTTCAC	173
Osteoprotegerin (*OPG*)	GGCGCTACCTTGAGATAGAGTTCTG; TGTTTTCTACAGGGTGCTTTAGATGAC	160
Receptor activator for nuclear factor kappa B ligand (*RANKL*)	CGTCGCCCTGTTCTTCTATTTC; AAATGCAGTGAGTGCCATCTTC	74
Low-density lipoprotein receptor related protein 5 (*LRP5*)	TACTGGACAGACTGGCAGACC; GTGTAGAAAGGCTCGCTTGG	209
Low-density lipoprotein receptor related protein 6 (*LRP6*)	TACTGGCCAAATGGACTGACT; TGTTGCAAGCCAAAATGGAGT	211
Dickkopf Wnt signaling pathway inhibitor 1 (*DKK1*)	ATGCGTCACGCTATGTGCT; GGAATACCCATCCAAGGTGCTA	144
Polymerase RNA II polypeptide A (*POLR2A*)	TTTTGGTGACGACTTGAACTGC; CCATCTTGTCCACCACCTCTTC	125

denaturation) and 65 °C for 30 s (primer annealing and extension). Results were analyzed with LightCycler 1.5.0 software (Roche Applied Science), using the second derivative method for cycle threshold calculations. Polymerase (RNA; DNA directed) II polypeptide A (POLR2A) was used as housekeeping gene.

Human subjects

Thirty-two volunteered elderly (10 males and 22 females) were recruited to participate in the study, through advertisement and verbal communication. The inclusion criteria, assessed during a screening visit, were: (1) Adults aged 75 years or older; (2) Subjects not living above 1500 meters above mean sea level during the last three months; (3) Free of illness or medication potentially affecting the bone system; (4) Estimated daily calcium intake of 500 mg/d or more; and (5) Consumption of no more than four alcoholic beverages per week. An exclusion criterion was a frequency of participation in the program < 80% (participants who missed > 20% of exposure sessions).

Twenty-four volunteers were informed about the study procedures and requested to sign a declaration. They voluntarily consented to participate in this research. Chosen volunteers were randomly divided into two groups. A total of 12 elderly were exposed to normobaric hypoxia (HYP). A group of 12 age-matched elderly served as control group (CON), not participating in any intervention. Both groups were instructed to continue with their normal daily activities for the duration of the study. Only the 10 individuals who completed the trial were included in the analyses. None

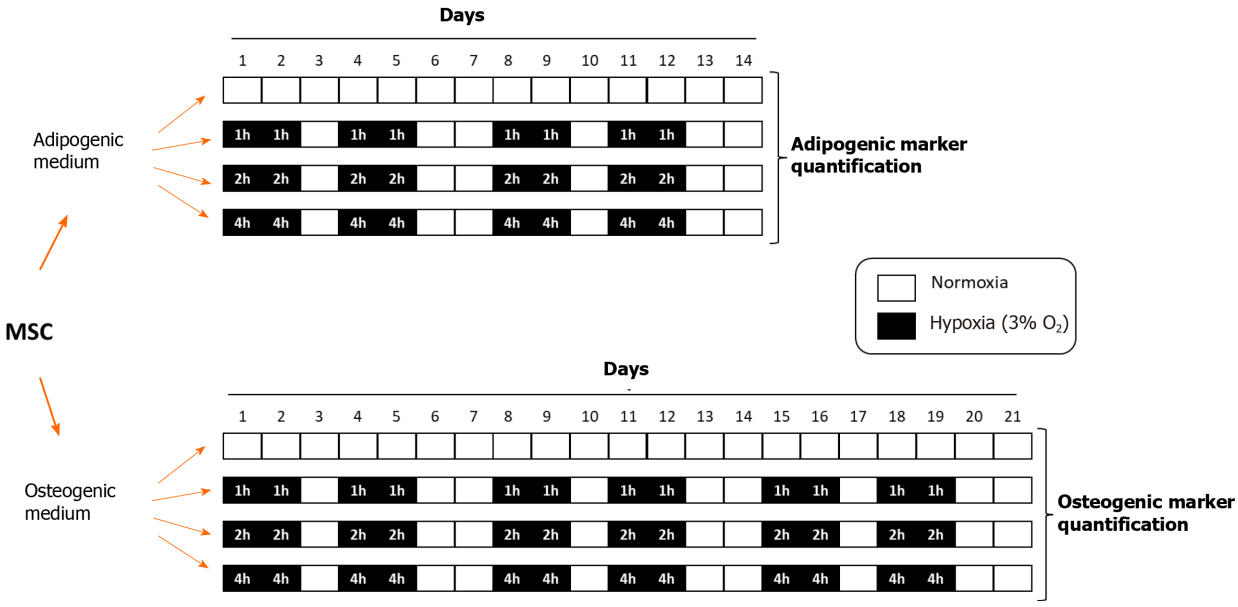

Figure 1 Temporal distribution of mesenchymal stem cells cyclic hypoxia-treatments. Mesenchymal stem cells induce to differentiate into adipocytes or osteoblasts were exposed to CH during 1, 2 or 4 h/d (3% O_2), four days per week. Cultures kept in continuous normoxia were use as controls. MSC: Mesenchymal stem cells.

of the dropouts left the program because of injuries or adverse responses to the exposure. All procedures were in accordance with the 1964 Helsinki declaration, later amendments and comparable ethical standards. The study was approved by the Bioethical and Biosecurity Commission of the University of Extremadura (17/2016).

Socio-demographic data and lifestyle questionnaires

A general questionnaire was administered to collect medical and demographic data, to check the inclusion criteria. Dietary energy, calcium and alcohol intake were estimated, using a semiquantitative food frequency questionnaire[28].

Experimental protocol

Volunteers completed 36 sessions, supervised by an experienced member of the research group, for 18 wk. The frequency of treatment was twice a week; sessions were scheduled with at least one day of rest in between, for optimal recovery. All patients were assessed at two time points by a group of researchers, who were blinded to the treatment assignment. Outcomes were measured at baseline (PRE) and after the last session (POST).

Hypoxia exposure

The HYP group performed an intellectual activity, while they were exposed to normobaric hypoxic conditions in a CAT 310 hypoxia chamber from Colorado Altitude Training (Lousiville, CO, United States), during 16 min/session. The fraction of inspired oxygen was set to 16.1%, in order to simulate an altitude of 2500 m above sea level. Saturation of peripheral oxygen and heart rate were monitored during testing, using a finger pulse-oximeter from Konica Minolta (Marunouchi, Chiyoda, Tokyo, Japan) and a Polar Team^2 heart-rate monitor from Polar Electro Oy (Kempele, Finland), respectively. This way, the physiological challenge posed on the participants during the exposure was controlled.

Timed up and go test

The timed up and go test was used to assess the functional mobility of subjects. From a sitting position, they had to stand up, walk 3 m, turn over, walk back and sit down again. The time needed to complete the task was recorded.

The body mass index and dual-energy X-ray absorptiometry assessment

The body mass index (BMI) was determined per the accepted method (BMI = weight/height^2, kg · m^{-2}). On the other hand, the percentage of fat mass and BMD values (g/cm^2), from whole body, trunk and right proximal femur (femoral, femoral

neck and trochanter), were assessed, using Norland Excell Plus dual-energy X-ray absorptiometry from Norland (Fort Atkinson, WI, United States). All dual-energy X-ray absorptiometry scans were performed by the same experimented technician, using the same device and software version, according to the manufacturer's guidelines. Quality assurance and calibration procedures were daily performed, prior to the scanning sessions. That ensured that no experimental machine bias occurred during the intervention period. A seca 225 portable stadiometer from seca (Hamburg, Germany) was used to measure height to the nearest 0.5 cm.

Statistical analyses

Statistical analyses were performed using SPSS v.20 for mac OS from IBM (New York, NY, United States). Standard statistical methods were used for the calculations of the mean and standard deviations. For the *in vitro* studies, experiments were carried out by triplicate, showing average ± standard error of the mean. Analysis of Variance and Fisher's projected least-significant difference tests were used to calculate P values. Differences were considered statistically significant when $P < 0.05$. For *in vivo* studies, Kolmogorov–Smirnov's tests were conducted to show the distribution of the analyzed variables. Additionally, the Levene's test was used for homogeneity of variance. The statistical significance of the difference for paired samples was estimated with Wilcoxon's test. On the other hand, the Mann–Whitney's test was used to check the differences between independent samples.

The effect size[29] was calculated for all variables, between baseline and after 18 wk of intervention. The magnitude of change considered was small (< 0.41), moderate (0.41 to 0.7) or large (> 0.7)[30]. Additionally, both the absolute and percentage changes from pre- to post-tests were calculated for all variables, for each group. The effects of the interventions were evaluated by pre-specifying the minimum detectable change (MDC). That is an absolute measure of reliability (measurement error), which accounts for various sources of variability, defining a confidence interval in units of the measure. These values are being increasingly used in scientific works. They assist in interpreting results, determining whether changes between repeated tests are due to random variations, or true changes in performance[31]. An effect was considered relevant when the change was greater than the MDC. Values of $P < 0.05$ were used as criterion for statistical significance.

RESULTS

The greater period of hypoxia during cyclic hypoxia decreases the ability of mesenchymal stem cells to differentiate into osteoblasts and adipocytes

Human MSC induced to differentiate into osteoblasts or adipocytes were exposed to four cycles of hypoxia per week, as described in materials and methods. In each cycle, cells were kept 1, 2 or 4 h in hypoxia (3% O_2). In osteoblast-induced cells, 4 h of exposure to CH inhibited extracellular matrix mineralization at day 21 (Figure 2A). Quantification of alizarin-red staining in osteoblast-induced cultures also showed that 2 h of hypoxia decreased mineralization, albeit the result was not statistically significant (Figure 2B). The hypoxia time in CH also affected the expression of the osteoblastic evaluated genes [Runt-related transcription factor 2 (RUNX2), Osterix (SP7), Collagen, type I, alpha 1 (COL1A1), Integrin-binding sialoprotein (IBSP) and BGLAP] (Figure 3). The *RUNX2* gene (encoding a transcription factor) increased its expression at day 7, in the cultures exposed to the longest hypoxia times (2 and 4 h). Yet, its expression did not vary significantly at day 13, with respect to the cultures maintained in normoxia.

The *SP7* gene (encoding the other transcription factor studied), did not vary its expression after 2 and 4 h of exposure to CH. But it decreased after one h in both times studied. The genes COL1A1 and IBSP (encoding for extracellular matrix proteins), were inhibited with CH exposure, mainly at day 13 and with the longest hypoxia times. Interestingly, IBSP expression decreased at day 6 and 13, in all hypoxia conditions tested (Figure 3). Osteocalcin is one of the most abundant noncollagenous proteins in the bone extracellular matrix. The expression of its encoding gene (BGLAP), unlike COL1A1 and IBSP, significantly increased at day 13 in cultures exposed to CH, with 2 and 4 h of hypoxia. On the other hand, the expression of the vascular endothelial growth factor A (VEGFA) gene, whose expression in hypoxia is induced through HIF-1A, was induced at day 13 in all CH exposed cultures; mainly with the longest hypoxia period (Figure 3).

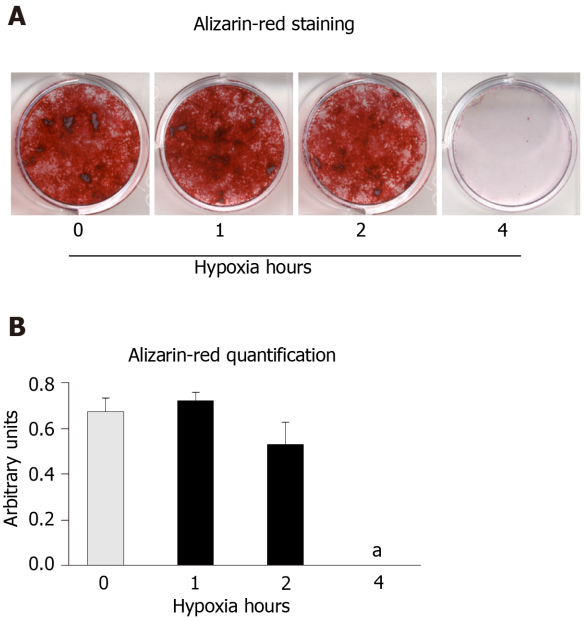

Figure 2 Increased hypoxia time in cyclic hypoxia inhibits extracellular matrix mineralization. Mesenchymal stem cells induced to differentiate into osteoblasts were exposed to cyclic hypoxia during 1, 2 or 4 h/d (3% O_2), four days per week. At day 21, the extracellular matrix mineralization of the cultures was stained with alizarin red. A: Representative images of alizarin-red staining of cultures differentiating into osteoblasts, in normoxia or cyclic hypoxia, after 1, 2 or 4 h of hypoxia; and B: Alizarin-red quantification. $^{a}P < 0.05$ *vs* normoxia (0 h of hypoxia).

Exposure to CH decreased the formation of lipid droplets in adipocyte-induced MSC, mainly in cultures exposed to 4 h of hypoxia (Figure 4). Additionally, the expression of adipogenic genes decreased in cultures exposed to the longest times of hypoxia; mainly at day 7 after adipogenic induction (Figure 5). The peroxisome proliferator-activated receptor gamma 2 (PPARG2) gene encodes the main transcription factor responsible for adipogenesis. Its expression was only significantly affected at day 13, in cultures exposed to 2 h of hypoxia during CH. However, the genes involved in fat metabolism, such as the ones encoding adipose triglyceride lipase (phospholipase domain-containing protein 2), fatty acid synthase and glycerol-3-phosphate dehydrogenase 1, showed the greatest decrease in expression in cultures exposed to CH, with periods of 2 or 4 h of hypoxia (Figure 5). Expression of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A) gene decreased at day 13, with 2 and 4 h of hypoxia in the CH. This gene is involved in energy metabolism and mitochondrial biogenesis. VEGFA expression significantly decreased at days 7 and 13 in cultures exposed to CH, mainly with 2 and 4 h of hypoxia (Figure 5).

Effect of cyclic hypoxia exposure on osteoprotegerin/ receptor activator for nuclear factor kappa B ligand expression in mesenchymal stem cells induced to differentiate into osteoblasts or adipocytes

The OPG/RANKL ratio is one of the main regulators of bone metabolism. Thus, its increase or decrease favors bone formation or resorption, respectively. Therefore, *OPG* and *RANKL* gene expressions were studied in MSC induced to differentiate into osteoblasts or adipocytes. MSC differentiating into osteoblasts decreased the expression of both genes at day 13, after exposure to CH with 4 h of hypoxia (Figure 6A). However, the OPG/RANKL ratio increased during this time, with the period of hypoxia in CH. It reached the maximum value in cultures exposed to 4 h of hypoxia (Figure 6A). OPG expression in MSC induced to differentiate into adipocytes increased at days 7 and 13, in cultures exposed to CH. The highest expression occurred in cultures subjected to 4 h of hypoxia (Figure 6B). In contrast, RANKL expression was significantly repressed at day 7 of adipogenic induction, in all hypoxia conditions tested. However, its expression was induced in cultures exposed to CH, with one h of hypoxia at day 13. No significant changes were observed in the other hypoxia conditions, with respect to the normoxia cultures (Figure 6B). Consequently, the OPG/RANKL ratio increased with CH exposure in the two times studied; mainly in cultures exposed to the longer hypoxia times (Figure 6B).

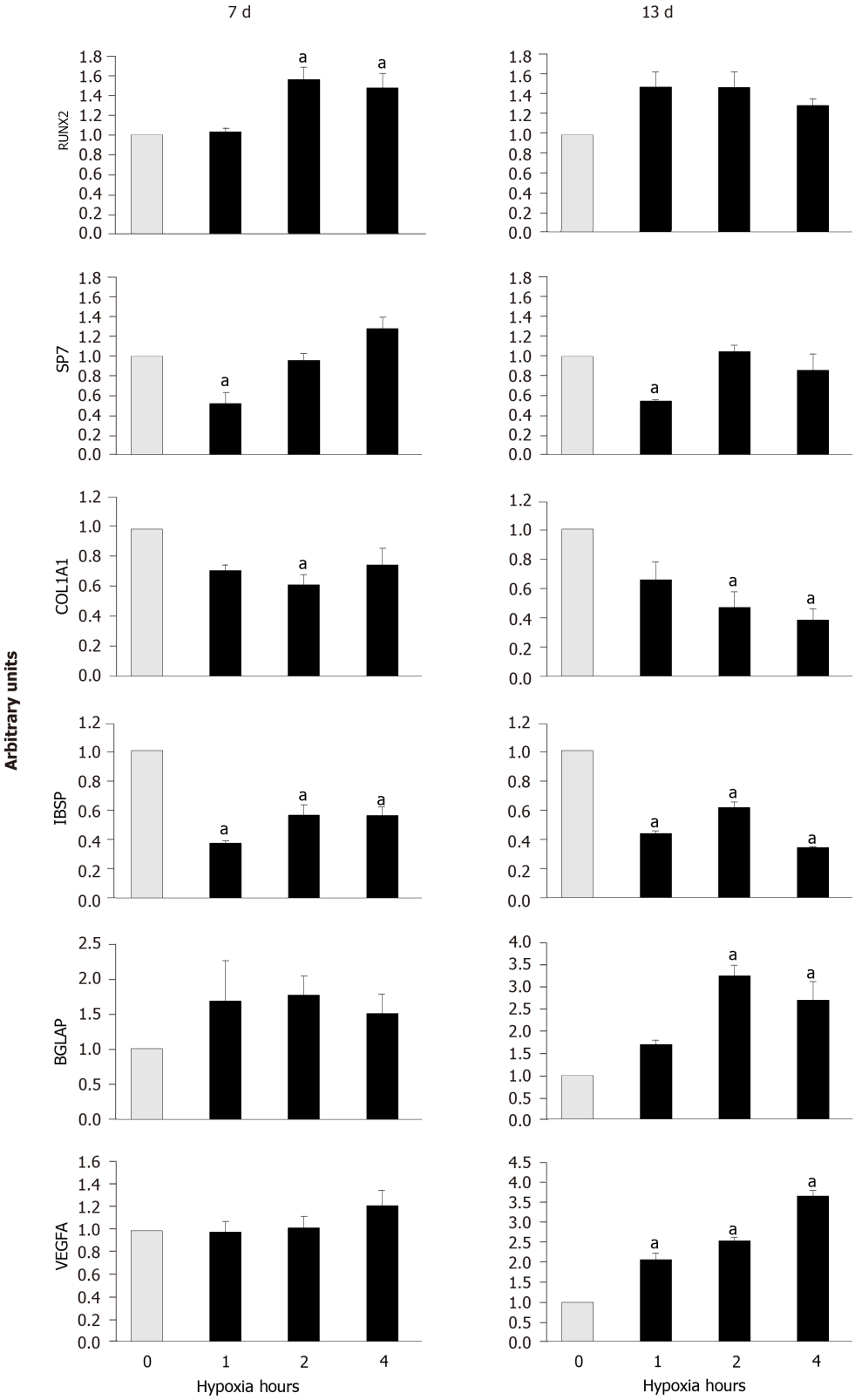

Figure 3 Quantification of gene expressions. The ones of osteoblastic markers (Runt-related transcription factor 2, Osterix, Collagen, type I, alpha 1, Integrin-binding sialoprotein and BGLAP) and Vascular endothelial growth factor A were measured in mesenchymal stem cells induce to differentiate into osteoblasts, in normoxia (0 hypoxia h) or cyclic hypoxia, at days 7 and 13 of osteogenic induction. [a]*P* < 0.05 *vs* normoxia (0 h of hypoxia). RUNX2: Runt-related transcription factor 2; SP7: Osterix; COL1A1: Collagen, type I, alpha 1; IBSP: Integrin-binding sialoprotein; and VEGFA: Vascular endothelial growth factor A.

Cyclic hypoxia affects differently the expression of dickkopf-1 in mesenchymal stem cells differentiating into osteoblasts or adipocytes

Dickkopf Wnt signaling pathway inhibitor 1 (DKK1) is a secreted inhibitor of β-catenin-dependent Wnt signaling. It does this by binding to low-density lipoprotein receptor related protein 5 or 6 (LRP5/6). This prevents their function as co-receptors, along with Frizzled protein family members, for transducing signals by Wnt and activating the β-catenin. Therefore, the relationship between the expression of LRP5/6 and DKK1 is considered an indicator of the activation status of β-catenin-dependent Wnt signaling. Interestingly, this pathway partly regulates the differentiation of MSC. Therefore, the expression of DKK1, as well as LRP5 and LRP6 (main LRP in osteoblasts and adipocytes, respectively) were studied. Experiments were carried out during the induction of osteogenic and adipogenic differentiation of MSC, under CH conditions. The expression of LRP5 in MSC differentiating into osteoblasts was only affected at day 13, in cultures exposed to 2 h of hypoxia. On the other hand, DKK1 expression decreased in cultures maintained in hypoxia for 1 and 4 h during CH, at days 7 and 13, respectively (Figure 7A). Thus, the ratio of LRP5/DKK1 expression increased at day 7, in cultures exposed to 1 h of hypoxia. A tendency to increase was observed in cultures exposed to CH, which was statistically significant in the treatment with 4 h of hypoxia, at day 13 (Figure 7A).

LRP6 expression showed little variation in CH-exposed cultures, compared to those maintained in normoxia, in MSC differentiating into adipocytes. However, the expression of DKK1 increased with CH, in direct proportion to the period of hypoxia, at days 7 and 13 (Figure 7B). Consequently, the LRP6/DKK1 ratio decreased in all cultures in CH, at day 7. This ratio increased in cells exposed to CH, with 1 h of hypoxia, at day 13. A reduction tendency was observed in cultures exposed to 2 and 4 h of hypoxia, with respect to normoxic cultures, albeit not being statistically significant (Figure 7B). Therefore, these results show that CH exposure of MSC, induced to differentiate into osteoblasts, tends to downregulate the expression of DKK1 and upregulate the LRP5/DKK1 ratio. Interestingly, the same conditions in MSC induced to differentiate into adipocytes produced the inverse effect.

Characteristics of human subjects

Functional and anthropometric characteristics of the 10 healthy elderly studied are presented in Table 2. No significant differences between groups were observed for any variable. Participants in both groups were independent (fit) elderly, since they performed the TUG test in the less than 20 s[32]. Not statistically significant differences ($P > 0.05$) were noted before and after the intervention, for habitual energy intake in CON (1784 ± 353.81 kcal/d *vs* 1770 ± 404.62 kcal/d for PRE and POST, respectively) nor HYP (2000 ± 71.69 kcal/d *vs* 1959 ± 44.31 kcal/d for PRE and POST, respectively) groups. Similar results were also found for estimated calcium intake ($P > 0.05$) in both groups between PRE (1263 ± 249.89 mg/d *vs* 1914 ± 71.69 mg/d for CON and HYP, respectively) and POST (1203 ± 268.89 mg/d *vs* 1908 ± 44.30 mg/d for CON and HYP, respectively) interventions. In the same way, not statistically significant differences between groups were observed.

Dual-energy X-ray absorptiometry assessment

Results of body composition and bone parameters are showed in Table 3. Neither whole body nor trunk fat mass significantly changed in both groups. Whole body BMD in HYP group showed a significant increase after hypoxia exposure, compared to CON group, whose values decreased ($P = 0.008$). Analyses within groups showed that HYP significantly increased whole body BMD ($P = 0.004$), whereas CON significantly decreased it ($P = 0.004$; d = 1.18). HYP group showed a significant decrease in the proximal femur, after intervention in the femoral neck ($P = 0.04$).

BMD responses were recorded after 18 wk (Figure 8). Parameter increases based on MDC were observed in different regions, following CH exposure. Thus, 20% of the subjects increased whole body (MDC = 0.104 g · cm^{-2}), right leg (MDC = 0.087 g · cm^{-2}) and femoral (MDC = 0.028 g · cm^{-2}) BMD. A total of 40% of the subjects of the hypoxia group also increased their trochanter BMD (MDC = 0.030 g · cm^{-2}). On the other hand, 80% of subjects in both groups decreased the femoral neck BMD, reaching the MDC (HYP = – 0.013 g · cm^{-2}; CON = – 0.016 g · cm^{-2}). The control group also decreased the rest of BMD values, reaching the MDC. They were 60% of the subjects in whole body (MDC = – 0.051 g · cm^{-2}), 40% in the right leg (MDC = – 0.049 g · cm^{-2}), 80% in the femoral (MDC = – 0.017 g · cm^{-2}) and 20% in the trochanter (MDC = – 0.030 g · cm^{-2}) BMD.

Table 2 Baseline characteristics of participants

Parameter	HYP (*n* = 5), mean ± SD	CON (*n* = 5), mean ± SD	*P* value
Age (yr)	83.00 ± 5.79	80.00 ± 4.06	0.371
Height (m)	1.61 ± 0.06	1.61 ± 0.06	0.960
Weight (kg)	62.00 ± 7.35	65.50 ± 2.87	0.350
BMI (kg · m^{-2})	23.82 ± 2.68	25.26 ± 1.72	0.340
TUGT (s)	9.25 ± 0.87	10.47 ± 1.61	0.174
Diseases (*n*)	1.60 ± 1.52	2.20 ± 0.84	0.461
Medication (*n*)	5.60 ± 5.98	1.80 ± 0.45	0.194

HYP: Hypoxic exposure group; CON: Control group; BMI: Body mass index; TUGT: Timed up and go test.

Table 3 Dual-energy X-ray absorptiometry measurements at baseline (PRE) and after 18 wk (POST) for hypoxia exposure (*n* = 5) and control (*n* = 5) groups

Parameter		PRE, (mean ± SD)	%	POST, (mean ± SD)	Cohen's d measure	MDC (%)	Wilcoxon's test	Man Whitney *U*'s test
Fat mass (%)								
Whole body	HYP	31.74 ± 10.08	–5.23	30.08 ± 12.47	0.15	12.38	0.225	0.841
	CON	36.39 ± 6.32	–1.37	35.89 ± 5.87	0.08	4.18	0.225	
Trunk	HYP	25.13 ± 6.08	8.40	27.24 ± 12.27	0.23	34.74	0.500	0.841
	CON	33.45 ± 7.66	7.23	35.87 ± 9.08	0.29	33.27	0.893	
Bone mineral density (g · cm^{-2})								
Whole body	HYP	0.880 ± 0.145	7.04$^{\$}$	0.942 ± 0.205	0.35	11.42	0.043	0.008
	CON	0.986 ± 0.053	–6.29	0.924 ± 0.052	1.18	5.37	0.042	
Right leg	HYP	0.908 ± 0.149	0.44	0.912 ± 0.186	0.02	9.55	0.892	0.310
	CON	0.994 ± 0.071	–3.22	0.962 ± 0.068	*0.46*	4.98	0.109	
Femoral	HYP	0.744 ± 0.118	0.81	0.750 ± 0.126	0.05	3.79	0.705	0.095
	CON	0.844 ± 0.042	–1.18	0.834 ± 0.051	0.22	2.06	0.129	
Femoral neck	HYP	0.686 ± 0.106	–2.92	0.666 ± 0.108	0.19	1.96	0.041	1.000
	CON	0.752 ± 0.067	–2.39	0.734 ± 0.068	0.27	2.11	0.059	
Trochanter	HYP	0.568 ± 0.097	3.17	0.586 ± 0.093	0.19	5.20	0.109	0.056
	CON	0.654 ± 0.064	–1.22	0.646 ± 0.069	0.12	4.66	0.461	

HYP: Hypoxic exposure group; CON: Control group; %: Percentage of change between PRE and POST measurement outcomes; MDC: Minimum detectable change.

DISCUSSION

Results showed that mineralization and lipid-droplets formation were inhibited in MSC cultures exposed to CH. That was positively correlated to increasing exposure time to hypoxia, during the differentiation into osteoblasts or adipocytes, respectively, *in vitro*. It has been reported that bone formation decreased in sustained hypoxia[33]. Our data indicate that this may be due, in part, to the effect on MSC differentiation. It has been observed that spontaneous calcification in MSC cultures from rats after 21 d is inhibited when cells are maintained at 2% O_2[34]. Similar results have also been obtained when primary rat osteoblastic cells were induced to differentiate into osteoblasts in hypoxia[35]. However, *in vivo* experiments have shown that exposure of rats to cyclic hypoxia for 5 h/d, 5 d/wk for 5 wk, increased bone mineral density with respect to rats maintained in normoxia[13].

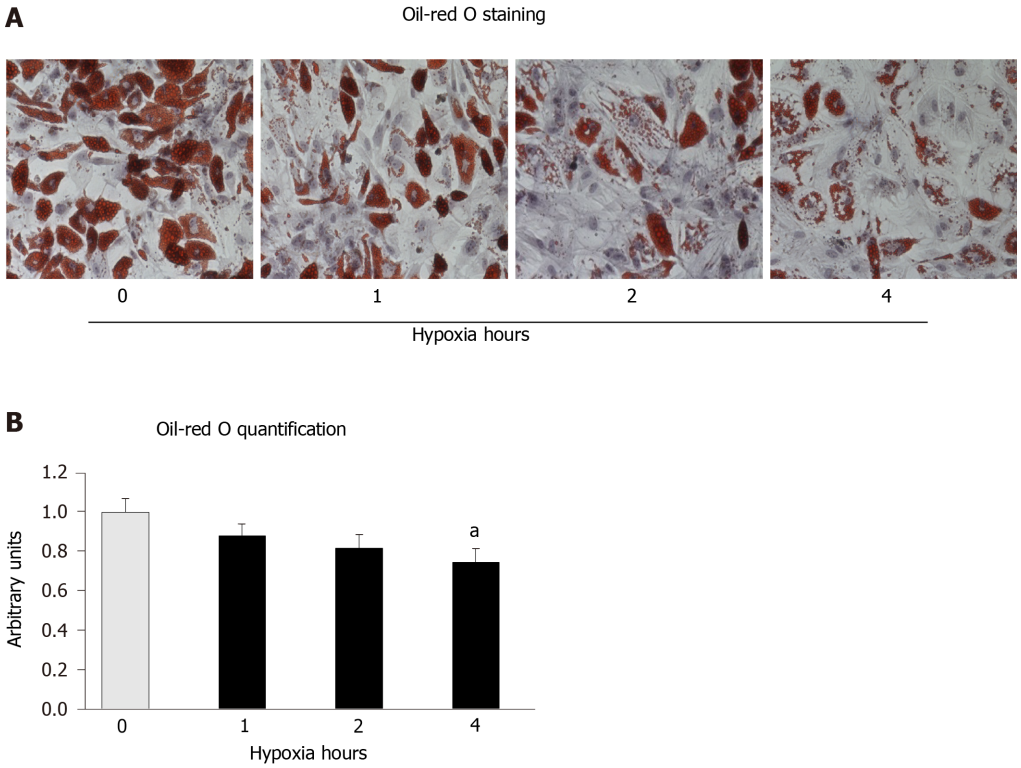

Figure 4 Increased hypoxia time in cyclic hypoxia inhibits lipid-droplet formation. Mesenchymal stem cells induced to differentiate into adipocytes were exposed to cyclic hypoxia during 1, 2 or 4 h/d (3% O_2), four days per week. Cultures were stained with oil-red O to reveal lipid droplets at day 13. A: representative images of oil-red O staining of cultures differentiating into adipocytes, in normoxia or cyclic hypoxia with 1, 2 or 4 h of hypoxia; and B: Oil-red O quantification. $^{a}P < 0.05$ *vs* normoxia (0 h of hypoxia).

Our data indicate that 4 h/d, 4 d/wk hypoxia inhibited osteoblast mineralization. This supports existing data on the negative effect of hypoxia on osteoblastogenesis. However, one- and two-h exposures did not significantly affect it. Therefore, the possible positive effect of cyclic hypoxia on bone mineral density, *in vivo*, may be due more to the inhibitory effect on osteoclastogenesis than to the activating effect on osteoblastogenesis, as previously suggested[13]. Thus, it has been described that osteoclastogenesis is induced under conditions of hypoxia[36]. Nevertheless, recent results have been reported, showing that constant conditions of hypoxia (1% O_2) inhibit osteoclastogenesis[37]. Such data controversy may be related to the fact that induction of HIF-1A in hypoxia may reduce osteoclast differentiation, yet enhancing bone resorption by mature osteoclasts[38], as well as different experimental conditions.

Analyses of gene expression showed that mRNA levels of two osteoblastogenesis-inducing transcription factors were not repressed in CH conditions. There are conflicting data in the literature on RUNX2 expression in hypoxia. Thus, while some authors describe an upregulation and osteogenic differentiation[39], others have shown that hypoxia inhibits osteoblastic differentiation, through RUNX2 downregulation; and that overexpression of HIF-1A in MSC also represses expression of the former[40-42]. Unlike these studies, there was cell culture reoxygenation after hypoxia in our experiments. That could prevent the repression of RUNX2, and consequently also of SP7. However, the expression of genes coding for extracellular matrix proteins (such as COL1A1 and IBSP), decreased with the hypoxia time in our conditions. Interestingly, it has been described that the expression of both COL1A1 and RUNX2 decreased under continuous hypoxia[34].

Therefore, our results suggest that the increase in the hypoxia period, during CH treatment, negatively affected the maturation of osteoblasts. Thus, MSC were maintained in a pre-osteoblast state, without the ability to mineralize. Indeed, it has been reported that pre-osteoblasts subjected to hypoxia did not mature, nor mineralize[43]. Thus, while pre-osteoblasts maintained at 2% O_2 for two weeks, and changed to 20% O_2 during the third week, do not mineralize, mineralization occurs after changing from 20% to 2% O_2[43]. It has also been observed that the induction of HIF-1A upregulates the metastasis-associated gene 1. In turn, this increased the proliferation of mouse osteoblast precursor cells (MC3T3), but inhibited their differentiation and mineralization[44]. In this sense, although the effect of hypoxia on

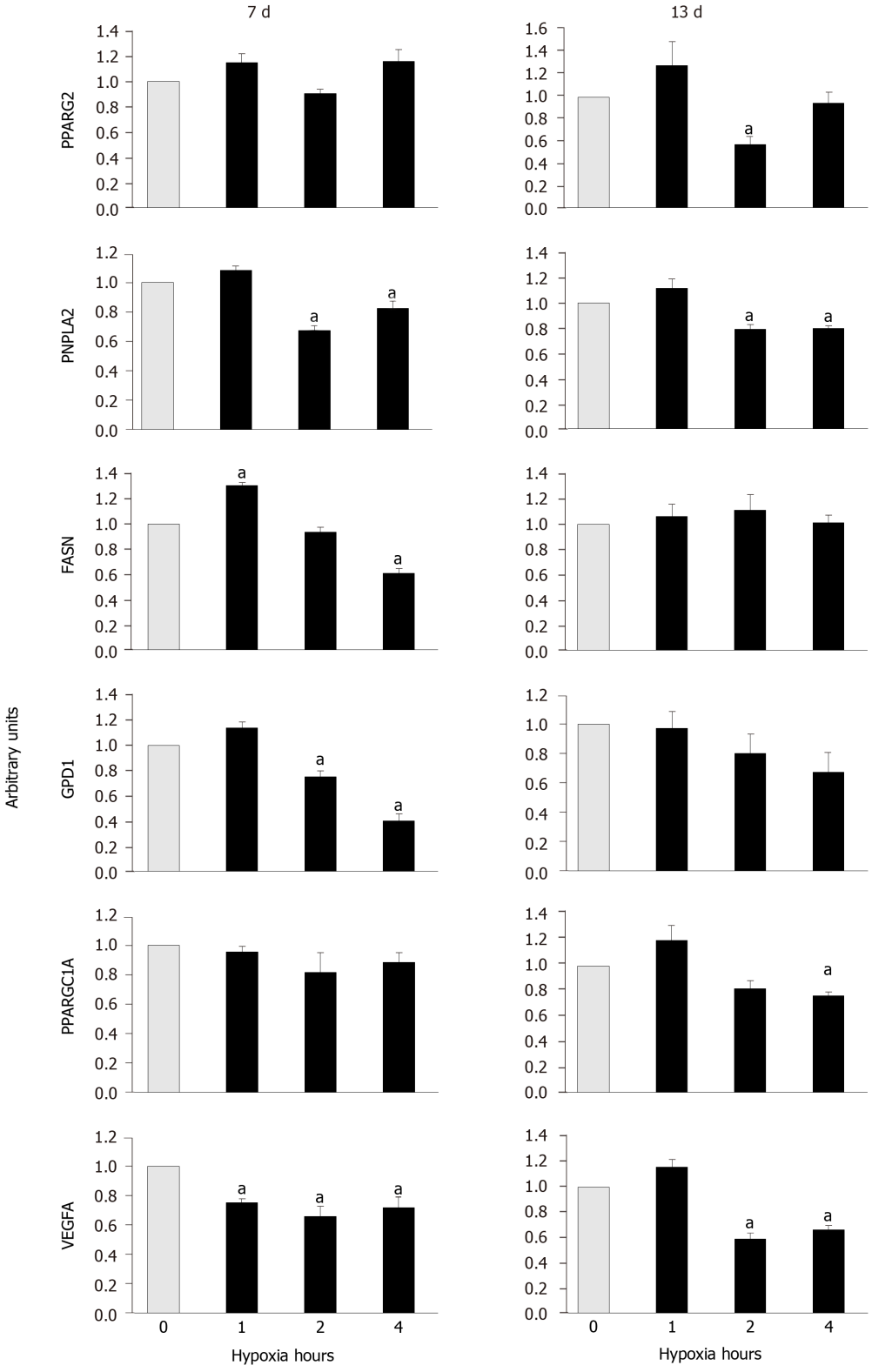

Figure 5 Expressions of adipogenic marker and vascular endothelial growth factor A genes. The ones encoding adipogenic markers (Peroxisome proliferator-activated receptor gamma 2, patatin-like phospholipase domain-containing protein 2, Fatty acid synthase, Glycerol-3-Phosphate Dehydrogenase 1 and Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha) and vascular endothelial growth factor A were measured in mesenchymal stem cells induce to differentiate into adipocytes. Experiments were carried out in normoxia (0 hypoxia h) or cyclic hypoxia, at days 7 and 13, after adipogenic induction. $^{a}P < 0.05$ *vs* normoxia (0 h of hypoxia). VEGFA: Vascular endothelial growth factor A; PPARG2: Peroxisome proliferator-activated receptor gamma 2; PNPLA2: Patatin-like phospholipase domain-containing protein 2; FASN: Fatty acid synthase; GPD1: Glycerol-3-phosphate dehydrogenase 1; and PPARGC1A: Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha.

osteoblast differentiation *in vitro* is not completely defined, it is generally accepted that hypoxia favors the maintenance of cells in an undifferentiated state[45,46].

The glucose metabolism of undifferentiated MSC is mainly based on glycolysis. However, oxidative phosphorylation is activated during mineralization, taking place when differentiating from pre-osteoblasts into osteoblasts[47]. The induction of HIF-1A favors glycolysis. Therefore, MSC induced into osteoblasts in hypoxia remained undifferentiated[47]. Thus, the 4 h of hypoxia in CH of our model seems enough to maintain cells in a pre-osteoblastic state. This effect may be mediated by the increase in the LRP5/DKK1 ratio, observed mainly at day 13 of osteogenic differentiation. Interestingly, LRP5 encodes a membrane co-receptor that interacts with the frizzled-4 receptor and is involved in the activation of Wnt/β-catenin pathway. On the other hand, DKK1 encodes an inhibitor of the pathway, which acts by binding and neutralizing LRP5 and LRP6 proteins. Therefore, the relationship between LRP5 and DKK1 partly modulates the activity of the Wnt/β-catenin pathway[48]. Activation of this pathway is necessary when precursor cells differentiate into pre-osteoblasts. However, the Wnt/β-catenin pathway must be inhibited for maturation of osteoblasts into osteocytes[49]. Also, it has been described that the expression of RUNX2 must decrease in the last phases of differentiation, for correct progression of osteoblastic differentiation[50].

Increasing the period of hypoxia in CH suppresses mineralization of MSC induced to differentiate into osteoblasts, in our experiments. That can be explained by the maintenance of the expression of RUNX2 and activation of the Wnt/β-catenin pathway, preventing progression from pre-osteoblasts to mature osteoblasts. It has been reported that induction of HIF-1A in MC3T3 cells decreases the activity of the Wnt signaling pathway. That happens through the upregulation of sclerostin (mainly expressed by osteocytes), which encodes an inhibitor of such pathway[51]. It has also been reported that hypoxia and the stabilization of HIF-1A downregulate the expression of sclerostin and DKK1, using a different model in vitro and *in vivo*. That increases the activity of the Wnt/β-catenin pathway[52,53]. The results in our CH model, although using a different exposure to hypoxia, are partially in agreement with the data of such latest studies.

Surprisingly, the osteocalcin-encoding gene increased its expression in cultures exposed to longer hypoxia, unlike COL1A1 and IBSP. Osteocalcin is the most abundant non-collagenous bone matrix protein. It modulates the osteogenic differentiation of MSC, playing an essential role in bone mineralization[54]. But it is also considered a multifunctional hormone produced by bone, modulating the regulation of the glucose metabolism. Thus, it has been described to enhance the glucose uptake in muscle, insulin production in pancreas, insulin sensitivity in liver, promotion of β-cell proliferation in pancreas and adipose tissue upregulation of adiponectin expression[55,56]. Recently, however, new findings have questioned such putative hormonal function of circulating osteocalcin, and thus its role on the energy metabolism, mainly in humans[57,58].

The higher expression of the osteocalcin gene in CH, with the period of hypoxia, cannot be related to an increase in mineralization in our model. The rationale is that a significant decrease of extracellular matrix mineralization was observed in these cultures. Therefore, a possible explanation is that the observed increase in expression is part of the metabolic response of osteoblasts to CH. Indeed, the glucose metabolism by osteoblasts is primarily glycolytic. That is less efficient than oxidative phosphorylation: 2 *vs* 38 ATP moles per metabolized glucose mole, respectively[59]. The decrease in O_2 levels in hypoxia favors glycolysis through HIF-1A, which is further stabilized by osteocalcin, leading to an increased rate of glucose uptake. In other words, osteocalcin favors the glycolytic pathway against the mitochondrial oxidative phosphorylation[60]. Therefore, the interaction between HIF-1A and osteocalcin could explain our results, although further research would be needed to obtain more conclusive results in this regard.

Additionally, CH with the longest period of hypoxia decreased the formation of lipid droplets, downregulating the expression of genes involved in lipid metabolism, in MSC induced to differentiate into adipocytes. This suggests that hypoxic treatments may keep cells in undifferentiated stages, as in MSC induced to differentiate into osteoblasts. Hypoxia induces genes involved in glucose metabolism in adipocytes, including the ones encoding transporters (like GLUT1, GLUT3 and GLUT5). Additionally, it represses the expression of genes related to adipogenesis, mitochondrial biogenesis (such as PPARG2 and PPARGC1A) and oxidative phosphorylation[61]. During adipogenesis, changes in gene expression allow to adapt the cell metabolism to oxidative phosphorylation, involving structural and functional changes in mitochondrial content[62-64]. This adaptation occurs within the first seven

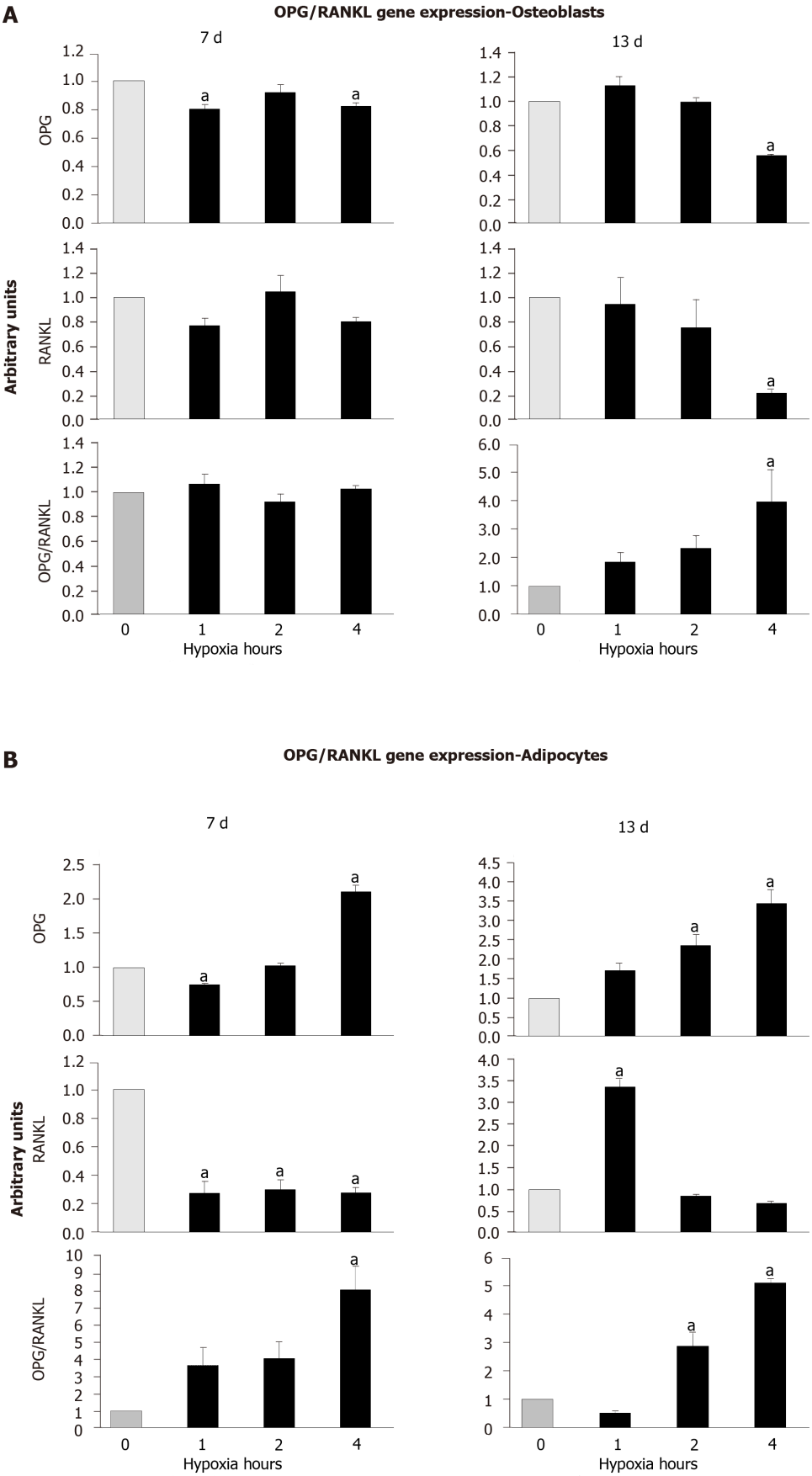

Figure 6 Expressions of osteoprotegerin and receptor activator for nuclear factor kappa B ligand genes. They were quantified at days 7 and 13 in mesenchymal stem cells differentiating into (A) osteoblasts or (B) adipocytes, in normoxia or different cyclic hypoxia conditions. $^{a}P < 0.05$ *vs* normoxia (0 h of hypoxia). OPG: Osteoprotegerin; and RANKL: Receptor activator for nuclear factor kappa B ligand.

days of adipogenic differentiation of human adipose tissue-derived stromal cells[65].

Therefore, our results indicate that 4 h of hypoxia during CH treatment produces changes in gene expression and metabolism, in MSC induced to differentiate into adipocytes. That includes downregulating the *PPARGC1A* gene. The rationale is that the glycolytic metabolism predominates during the hypoxia period. This inhibits the maturation of adipocytes, because it negatively interferes with the change towards an oxidative metabolism, based on greater mitochondrial activity, as well as an important role of oxidative phosphorylation. Our results agree with those observed in murine C3H/10T1/2 MSC. Thus, such cells lose capacity to differentiate into adipocytes after being pre-conditioned for 24 h with 0.1 mmol/L $CoCl_2$, mimicking low-oxygen conditions[66].

Interestingly, CH increased the expression of the DKK1 gene and decreased the LRP6/DKK1 ratio, proportionally to the time of hypoxia, in MSC induced to differentiate into adipocytes. That should inhibit the Wnt/β-Catenin pathway, consequently enhancing adipogenesis[67]. However, contrary to expectations, the cultures with a higher induction of DKK1 were those with lower adipogenesis. Interestingly, DKK1 is mainly expressed in the early stages of adipogenesis, decreasing during the final maturation of adipocytes[68,69]. It has been recently described that mature adipocytes express various components of the Wnt/β-Catenin pathway. Additionally, they intervene in the regulation of lipogenesis, as well as the expansion of white adipose tissue[70,71]. Therefore, a possible explanation for the results is that the continuous upregulation of DKK1, with the greatest periods of hypoxia, prevents the correct maturation of adipocytes. In this regard, other authors have shown that MSC exposed to hypoxia increased the expression of DKK1[72], and that such gene modulates signaling pathways, promoting cell proliferation and migration[73]. Therefore, it would be interesting to further explore how its overexpression may affect both preadipocytes and mature adipocytes.

CH also differently affected the expression of VEGFA in osteoblastic differentiation, in relation to the adipocytic one. While its expression increased in the former, it decreased in the latter, mainly with longer hypoxia times. Generally, hypoxia stimulates the expression of VEGF, through HIF-1A, working as an angiogenic factor in response to low O_2 levels. Additionally, RUNX2 and HIF-1A interact to induce VEGF expression in osteoblasts[74]. That is in agreement with our results. On the other hand, exposure to hypoxia should induce VEGF in adipocytes[61]. Yet, treatment of cyclic hypoxia repressed it. One possible explanation is that the increase of DKK1 expression negatively affect the Wnt/β-Catenin pathway. As this pathway may directly (or by interacting with HIF-1A) induce VEGF transcription, its inhibition may affect VEGF expression[75,76].

One of the most interesting effects of CH on both osteoblastogenesis and adipogenesis was the increase of the OPG/RANKL ratio. Such genes encode factors that are part of the regulation of bone remodeling. The RANKL protein binds to its RANK receptor in cells of the osteoclastic lineage, activating osteoclastogenesis and bone resorption. On the other hand, OPG is a decoy receptor of RANKL, preventing its binding to RANK, and therefore osteoclastic activation[7]. Interestingly, HIF-1A can induce OPG expression[12]. Thus, overexpression of HIF-1A in osteoblasts increased bone mass in mice, through a decrease in the resorptive activity of the osteoclasts. That is a consequence of the upregulation of OPG[77]. Our data showed that CH in osteoblasts did not induce the expression of OPG but increased the OPG/RANKL ratio. It is described that the expression of OPG increase with the maturation of osteoblasts but it is repressed in pre-osteoblasts that constitutively express RUNX2, which prevents their correct maturation[78,79]. Therefore, our data suggest that MSC induced to differentiate into osteoblasts remain in a more undifferentiated state in CH, with hypoxia times of 4 h. This may negatively affect the expression of OPG. However, our data did not necessarily mean that CH activated osteoclastogenesis. The rationale is that RANKL expression also decreased, as described by some authors for longer or sustained periods of hypoxia[80,81].

On the other hand, OPG and RANKL expressions increased and decreased, respectively, in MSC induced to differentiate into adipocytes, with CH, mainly with longer periods of hypoxia. The expression of such genes in adipose tissue has been described[82-84]. In addition, RANKL expression by bone marrow adipocytes is involved in the regulation of bone remodeling. Thus, bone resorption increased with bone-marrow adipocytes that secrete RANKL, in type I diabetic mice[85]. Ovariectomy also increased the expression of RANKL in bone-marrow adipocytes. This may contribute to the loss of bone mass[86].

The differentiation of MSC from bone marrow into adipocytes increased with age. As a consequence, the formation of new osteoblasts decreased, which favors the loss of

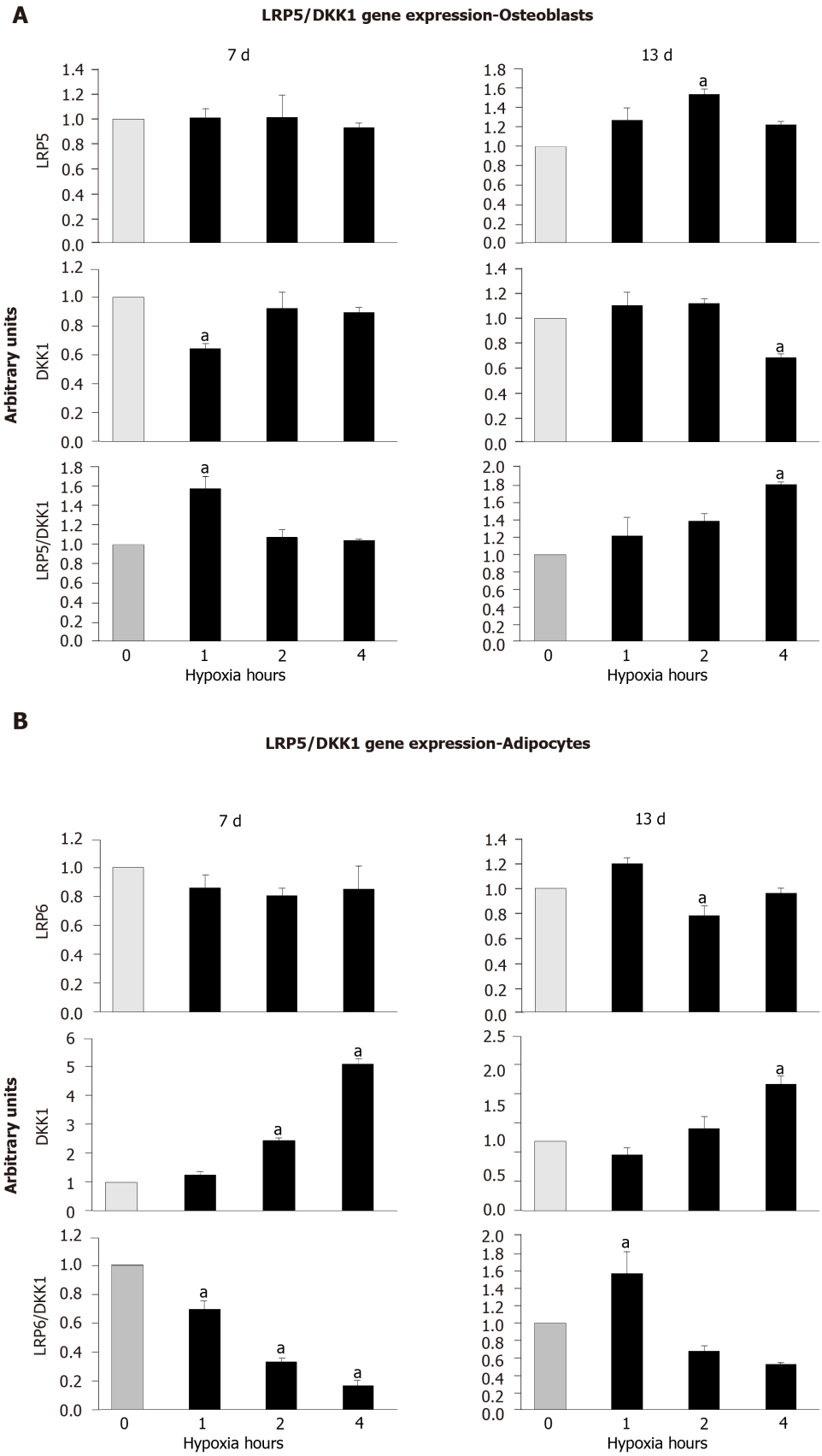

Figure 7 Expressions of low-density lipoprotein receptor related protein 5, low-density lipoprotein receptor related protein 6 and Dickkopf Wnt signaling pathway inhibitor 1 genes. They were quantified at days 7 and 13, in mesenchymal stem cells differentiating into (A) osteoblasts or (B) adipocytes, exposed to normoxia or different cyclic hypoxia conditions. [a]*P* < 0.05 *vs* normoxia (0 h of hypoxia). DKK1: Dickkopf Wnt signaling pathway inhibitor 1; LRP6: Low-density lipoprotein receptor related protein 6; and LRP5: Low-density lipoprotein receptor related protein 5.

bone mass and the development of osteoporosis[4]. In this context, it has been described that pre-adipocytes express RANKL in the adipose tissue of bone marrow. That directly contributes to the osteoclastic activity and bone resorption, which is associated to aging[84,87]. Our results showed that CH exposure can reverse the induction of RANKL expression, in the early stages of adipogenesis, while inducing OPG expression, in MSC induced to differentiate into adipocytes. This suggests that exposure to CH may raise the OPG/RANKL ratio in bone marrow, partly preventing age-related bone mass loss. This is particularly relevant in older people, who have higher bone marrow adiposity, and therefore higher RANKL secretion by adipocytes.

The aim of this pilot study in humans was to investigate the effects of CH, during 18 wk, on BMD and fat mass of elderly adults. Cyclic exposure to moderate hypoxia was sufficient to increase whole body BMD. Yet, it did not alter femur proximal BMD, nor fat mass percentage. Results of this investigation showed that the fat mass percentage in healthy elderly adults did not decrease, using eighteen 16-min sessions of CH exposure (16.1% O_2). Yet, previous studies have showed decreases in body fat, after hypoxia exposure in different conditions. This might be due to the profile of body composition, varying with the hypoxia duration or severity[88]. Natural high altitude or artificial hypoxic conditions enhanced the oxygen transporting ability of muscles. That improved fat oxidation, reducing body fat[88,89].

Some studies have shown that moderate hypoxia could involve higher fat mass reduction[90]. However, such effects could be due to differences between the studied populations[91] or experimental design. It has been reported that obese subjects are three times more susceptible to the hypoxia effects that people with normal BMI[92]. Furthermore, inter-individual variability and confounding variables of fit or normal-weight participants may bias results. Among them are exercise or fitness, which might have interfered with hypoxic exposure results[89]. Therefore, a moderate CH exposure of 18 wk could not be sufficient to provoke changes in fat mass percentages, in subjects with normal BMI. Longer lasting or more severe passive hypoxia (until 12% O_2) could be necessary to improve the body composition, such as our *in vitro* results suggest on the effect of CH on MSC differentiating into adipocytes. Total BMD significantly increased compared to the control group, whose levels decreased after exposure to CH (twice a week, 16 min per session, for 18 wk), simulating 2500 m above sea level. Although the results indicated that it might cause an individualized response after 18 wk of treatment, such exposure was not sufficient to improve the proximal femoral BMD.

Few studies have investigated the *in vivo* effect of CH on BMD. Different findings have been reported, after applying passive CH protocols in rats. For instance, healthy rats were exposed to CH during 2 wk (4 h per day, simulating 3000 to 5000 m above sea level), maintaining BMD throughout the body[80]. In another study, increases in dorsal spine BMD were observed after 5 wk of exposure to CH (5 d per week, 5 h per day) at 4500 meters above sea level[13]. In this way, the number of episodes of hypoxia, severity and total duration of exposure can lead to different physiological responses. Therefore, a certain number of short episodes with a modest level of hypoxia (9%-16% of O_2) could generate benefits, if repeatedly administered for weeks[93].

CONCLUSION

Exposure to CH may have an impact on bone health because it affects the osteogenic and adipogenic differentiation of MSC. Thus, CH tends to inhibit differentiation to both cell types, increasing the OPG/RANKL expression ratio, although requires longer hypoxic periods. Therefore, our results suggest that CH could be applied to improve bone health, decreasing resorption mainly in the elderly, as has been suggested by other authors[13,33]. The *in vitro* results obtained may partly explain those obtained in our pilot study, conducted on elderly people. Our *in vivo* results indicate that exposure to CH (16.1% O_2 for 18 wk) is sufficient to significantly increase total BMD in older adults, but not in specific regions, such as the proximal femoral. This passive protocol is also not enough to decrease the percentage of fat mass. In summary, CH exposure is a potential tool for the prevention and treatment of age-related dysfunctions once an optimal protocol is defined. Indeed, the available evidence is promising, albeit limited. Therefore, larger randomized controlled trials are required to ascertain the significance of current results.

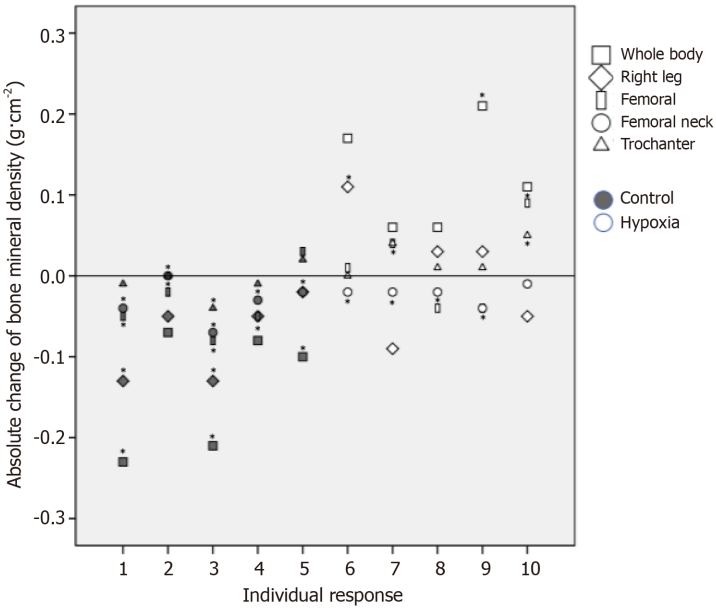

Figure 8 Individual response of bone mineral density. Changes were recorded after 18 wk of normal daily activities (control) or hypoxia exposure. Intra-individual difference equal or greater than minimum detectable change (*i.e.*, 0.10 g · cm^{-2} of whole-body bone mineral density in relation to hypoxia group).

ARTICLE HIGHLIGHTS

Research background

Bone mass and strength decline with aging. Mesenchymal stem cells (MSC) are precursors of osteoblasts and adipocytes. Osteoblastogenesis decreases, and adipogenesis increases, in bone-marrow MSC with aging. Receptor activator for nuclear factor kappa B ligand (RANKL) induces osteoclastogenesis, whereas Osteoprotegerin (OPG) represses it. Therefore, the OPG/RANKL ratio regulates bone resorption. Hypoxia induces the *HIF-1A* gene, encoding a transcription factor which regulates, among others, genes involved in angiogenesis and osteogenesis. Chronic hypoxia has negative effects on bone. Yet, cyclic exposure to hypoxia for short periods, followed by long times in normoxia, may have beneficial effects.

Research motivation

Cyclic hypoxia can be a non-pharmacological method for the prevention and treatment of different clinical conditions, such as loss of bone mass with age.

Research objectives

Investigate the effects of cyclic hypoxia exposure on differentiation of human MSC, derived from bone-marrow, into osteoblasts or adipocytes. Conduct a pilot study on the cyclic hypoxia effect on bone-mineral density and fat mass in elderly.

Research methods

MSC were induced to differentiate into osteoblasts or adipocytes, in cyclic hypoxia (3% O2 for 1, 2 or 4 h, 4 d a week). Osteogenic and adipogenic markers were measured. In addition, elderly were exposed to either low oxygen concentration in air (simulating an altitude of 2500 m above sea level) in a hypoxia chamber, or to normoxia, for 18 wk (36 CH sessions of 16 min each). Percentages of fat mass and bone mineral-density from whole body, trunk and right proximal femur were assessed, using dual-energy X-ray absorptiometry.

Research results

Cyclic hypoxia with 4 h of hypoxia exposure inhibited osteogenesis and adipogenesis. Osteocalcin, vascular endothelial growth factor A and LRP (5/6)/ dickkopf-1 gene expressions were upregulated in osteoblasts. Yet, the latter decreased in adipocytes. Cyclic hypoxia treatments increased the OPG/RANKL ratio, in both cell types. Elderly exposed to cyclic hypoxia increased total bone mineral-density, although the percentage of fat did not vary between groups.

Research conclusions

Cyclic hypoxia affects MSC differentiation into osteoblasts or adipocytes. It can increase bone mass, by inhibiting osteoclastic activity, through upregulating the OPG/RANK ratio.

Research perspectives

The potential of using cyclic hypoxia to prevent and treat bone-mass loss associated with ageing is promising. Yet, its mechanisms of action need to be further explored. Trials with a larger number of participants should be carried out to evaluate different patterns and times of exposure to cyclic hypoxia.

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