Serum from postmenopausal women treated with a by-product of olive-oil extraction process stimulates osteoblastogenesis and inhibits adipogenesis in human mesenchymal stem-cells (MSC)

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ABSTRACT

Aging may enhance both oxidative stress and bone-marrow mesenchymal stem-cell (MSC) differentiation into adipocytes. That reduces osteoblastogenesis, thus favoring bone-mass loss and fracture, representing an important worldwide health-issue, mainly in countries with aging populations. Intake of antioxidant products may help to retain bone-mass density. Interestingly, a novel olive-pomace physical treatment to generate olive oil also yields by-products rich in functional antioxidants. Thus, diet of postmenopausal women was supplemented for two months with one of such by-products (distillate 6; D6), being rich in squalene. After treatment, serum from such women showed reduced both lipidic peroxidation and oxidized low-density lipoprotein (LDL). Besides, vitamin E and coenzyme Q10 levels increased. Furthermore, culture medium containing 10% of such serum both increased osteoblastogenesis and reduced adipogenesis in human MSC from bone marrow. Therefore, highly antioxidant by-products like D6 may represent a relevant source for development of functional products, for both prevention and treatment of degenerative pathologies associated with aging, like osteoporosis.

Key words: Olive-pomace oil distillate, mesenchymal stem-cells, osteoblasts, adipocytes, aging, osteoporosis.

1. INTRODUCTION

Age-related loss of bone-mass density (BMD) and strength are invariable features of human biology. They affect women and men alike, being determinant of osteoporosis and fracture risk (Drake et al., 2015). Such disease is associated to increased osteoclastogenesis levels, due to estrogen and vitamin-D deficiencies. It also shows osteoblast insufficiency during continuous bone remodeling, being more relevant in postmenopausal and elderly women (Raisz, 2005). Osteoblastogenesis decrease is associated with increasing bone-marrow adipogenesis. Indeed, increased bone-marrow adiposity accompanies osteoporosis in aging populations (Hartsock et al., 1965; Verma et al., 2002). Since osteoblasts and bone-marrow adipocytes are derived from common-multipotential mesenchymal stem-cell (MSC) progenitors, it has been hypothesized that the close relationship between these lineages underlies reciprocal relationships between increased adipocytes and decreased bone formation that occurs during aging (Chen et al., 2016). Several agents, including hormones and other metabolic signals, can switch pluripotent MSC differentiation toward adipocytes or osteoblasts. Basically, stimuli inducing osteoblast differentiation inhibit adipogenesis, whereas those inducing adipocyte differentiation inhibit osteogenesis (Chen et al., 2016).

Lineage commitment of MSC is determined by induction of gene expression and activation of specific transcription-factor proteins: runt-related transcription factor 2 (*RUNX2*) and osterix (*SP7*) in the case of osteoblasts; and CCAAT-

enhancer-binding protein beta (*C/EBP β*) and peroxisome proliferator-activated receptor gamma (*PPARG*) in the case of adipocytes (Chen et al., 2016). *PPARG* nuclear transcription-factor is activated by a variety of organic ligands. They include some generated by oxidative stress, as oxidized low-density lipoprotein (LDL) cholesterol (OxLDL), 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE), which derives from oxidation of linoleic acid (LA) and arachidonic acid (AA) (Klein et al., 2004), as well as nitric-oxide derivatives of linoleic acid (Schopfer et al., 2005). Oxidized lipids promote binding of *PPARG2* to β -catenin, reducing the latter levels, thus decreasing wingless-related integration site (Wnt)- β catenin pathway effects on osteoblast proliferation and differentiation, further stimulating osteoblast apoptosis (Almeida et al., 2009). Thus, oxidative stress promotes MSC differentiation into adipocytes, while inhibiting osteoblast formation. Not surprisingly, the latter are also more susceptible to oxidative stress than adipocytes (Brueedigam et al., 2010).

Accumulated oxidative damage and weakened antioxidative defense-systems with aging may cause disturbance in organism's redox balance (Pandey and Rizvi, 2010). Indeed, oxidative stress could provide the underlying basis for molecular alterations linking aging and age-related pathological processes, like osteoporosis (Manolagas, 2010; Zhang et al., 2011). In fact, correlation between oxidative-stress markers and BMD has been reported for postmenopausal women. Not surprisingly, osteoporotic women have lower activity of antioxidant

enzymes and higher concentration of products from lipid peroxidation (Ozgocmen et al., 2007). Indeed, estrogens protect against reactive oxygen-species (ROS), whereas their deficiency accelerates bone loss in postmenopausal women (Almeida et al., 2007). Actually, ROS production by osteoclasts and associated bone resorption may overcome cell antioxidant-capacities in pathological conditions. That may promote osteoblast and osteocyte apoptosis, consequently reducing bone-forming capacity (Sheweita and Khoshhal, 2007).

In fact, it has been suggested that oxidative stress might play a role in postmenopausal bone-loss, by generating a more oxidized bone microenvironment (Maggio et al., 2003; Muthusami et al., 2005). Yet, bone-marrow microenvironment in which MSC multiply and differentiate is complex, being difficult to study. So, one research approach is to consider that blood serum should be somewhat similar to such microenvironment. Actually, it has been observed that composition of serum varies in premenopausal versus postmenopausal women. Interestingly, increase of compounds derived from lipid peroxidation and loss of antioxidant activity in serum may cause increased adipogenic differentiation. That has been observed in conditionally-immortalized human osteoprogenitor (hOP)-7 cells, when incubated in the presence of 10% serum from premenopausal versus postmenopausal women (Stringer et al., 2007). In fact, studies with ovariectomized rats treated with natural products with high antioxidant-capacity may prevent bone loss (Noorafshan et al., 2015). Therefore, it would be interesting to develop highly-

antioxidant products as food supplements or pharmaceutical drugs, for prevention and treatment of aging-related bone-mass loss (Sheweita and Khoshhal, 2007).

Mechanical milling of olives generates virgin olive-oil and a solid pomace by-product known as 'alperujo'. The latter still contains valuable olive oil, which has been traditionally recovered by drying and hexane extraction. Yet, that cause loss of many interesting and biologically-active compounds, as well as generating unwanted compounds. Fortunately, a new physical methodology allows to recover olive oil from alperujo, without unwanted compounds and rich in functional compounds (Artacho del Pino, 1994). Likewise, orujo olive-oil must be refined in order to be edible. As before, chemical treatments discard most bioactive compounds (Hamm et al., 2013). Thus, a new refining physical-procedure has been also developed for such olive oil, in which free fatty-acids are discarded in successive distillation steps, in vacuum at high temperatures (Ruiz Mendez et al., 2010). That generates edible orujo olive-oil, which is enriched in triterpenic acids, as well as different interesting fractions resulting from successive distillations. One of them is known as distillate 6 (D6), with >15% unsaponifiable content, mainly made of squalene and sterols. The former is considered an important component of Mediterranean diet. That is due, among other effects, to its potential chemopreventive activity against cancer, being a potent scavenger of singlet oxygen and cardioprotective (Bhilwade et al., 2010; Ghanbari et al., 2012). Therefore, such D6 fraction is rich in bioactive

compounds and has the potential of being a functional dietary supplement, capable of delaying degenerative processes associated to aging. Thus, we have studied the effects of serum from postmenopausal women (before and after D6-supplement intake) on human MSC differentiation into osteoblasts or adipocytes.

2. MATERIALS AND METHODS

2.1. Postmenopausal-women treatment and serum analyses

Five postmenopausal and healthy women participated in this study, aged 65.2 ± 6.4 years. Women were referred by general practitioners to our outpatient clinic of the Mineral Metabolism Unit at “Reina Sofía” Hospital (Córdoba, Spain) to evaluate endocrine status, in the framework of a population breast-cancer screening program. This study was reviewed and approved by the local Research Ethics Committee. Signed written-informed consent was obtained from all participants prior to their involvement. Diet of women was supplemented with 8 ml D6 in 100 ml skim milk for two months. D6 contained 12.6% unsaponifiable components (7.2% squalene, plus sterols and aliphatic alcohols), 47.8% alkyl esters and 6.6% triacylglycerols with 26.3% acidity (Table 1). Blood was obtained from each woman pre- and post-D6 treatment in BD Vacutainer flasks from Becton, Dickinson and Company (BD; Franklin Lakes, NJ, USA). Serum was isolated from them, pre- and post-treatment

samples were independently pooled to generate two samples and stored at -80°C until needed for measurements or cell-culture supplementations.

Vitamin A (retinol), E (α -tocopherol) and D₃ (25 hydroxyvitamin D₃) serum levels were quantified by automated high-performance liquid chromatography (HPLC). Eluates were monitored by photodiode-array detector at three wavelengths, as we have previously described (Quesada et al., 2004). Coenzyme Q10 (CoQ10A or Q10, also known as ubiquinone-10) was determined by liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS), with electrospray ionization, as we have reported (Ruiz-Jiménez et al., 2007). Oxidative-stress biomarkers were quantified, including: i) reduced glutathione (GSH), using Bioxytech GSH-400 Assay; ii) lipid peroxidation (LPO) products including malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA) by Bioxytech LPO-586 Assay; iii) potential antioxidant (PAO) using Bioxytech AOP-490 Assay; all three from Oxis International (Portland, OR, USA); and iv) glutathione peroxidase (GPx), as described elsewhere (Flohé and Günzler, 1984). Absorbances were quantified with UV-1603 spectrophotometer from Shimadzu (Kyoto, Japan). Plasma OxLDL was measured by enzyme-linked immunosorbent assay (ELISA), using mAb-4E6 monoclonal-antibody from Mercodia (Uppsala, Sweden).

Table 1. Composition of D6 distillate (percentages).

Free fatty-acids	Triacylglycerols	Alkyl esters	Unsaponifiable matter
Oleic acid (26.3)	6.6	47.8	Squalene (7.2)
			Aliphatic alcohols (0.19)
			Total sterols (0.29)
		<i>Alkyl-ester fraction composition</i>	<i>Sterol Fraction composition</i>
		14:0-Ethyl	0.13
		16:0-Ethyl	12.37
		16:1-Ethyl	0.80
		18:0-Ethyl	0.65
		18:1-Ethyl	55.04
		18:2-Ethyl	1.90
		18:3-Ethyl	1.90
		20:0-Ethyl	0.64
		16:0-Methyl	2.37
		18:0-Methyl	0.48
		18:1-Methyl	11.23
		Others	12.50
			Cholesterol
			0.97
			Campesterol
			4.89
			Campestanol
			1.29
			Stigmasterol
			1.75
			Δ^7 -Campesterol
			0.70
			Clerosterol
			1.87
			β -Sitosterol
			82.61
			sitostanol
			2.30
			Δ^5 -Avenasterol
			2.24
			Δ^5 -24-Stigmastadienol
			1.38

2.2. MSC cultures

MSC used were from our research group's collection. They were previously obtained from healthy bone-marrow donors at "Servicio de Hematología" of "Hospital Universitario Reina Sofía" in Córdoba (Spain), after signed written-informed-consent. MSC isolation was carried out as we have previously reported (Casado-Díaz et al., 2008). Cryopreserved cells were thawed and seeded in 75 cm² flasks from Nalgene-Nunc - Thermo Fisher Scientific (Waltham, MA, USA). They were grown in Minimum Essential-Medium Alpha (MEM α) from Cambrex Bio Science - Lonza (Basel, Switzerland), containing 2 mM UltraGlutamine from the same manufacturer, 15% fetal bovine-serum (FBS) from Invitrogen - Life Technologies - 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, USA). Cultures were incubated at 37 °C with 95% humidity and 5% CO₂. Medium was changed at

three- to four-day intervals. Once cultures reached near 90% confluence, cells were detached with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) from Cambrex Bio Science - Lonza and seeded in culture plates using medium described above (but with 10% FBS), at a density of about 500 cells/cm². Cultures were maintained until confluence.

Then, medium was replaced with fresh one without bFGF and cultures divided into three: i) in presence of 10% FBS (O-FBS and A-FBS, for osteoblastic and adipogenic differentiation, respectively); ii) with 10% serum pool from pre-treated postmenopausal women (O-PreS and A-PreS, as above); and iii) 10% serum pool from post-treated postmenopausal women (O-PosS and A-PosS, likewise). Cells were maintained undifferentiated for each of such three cultures, or they were induced to differentiate into osteoblasts or adipocytes. Osteoblastic differentiation was induced with 10⁻⁸ M dexamethasone, 0.2 mM ascorbic acid and 10 mM β -glycerolphosphate. Adipogenic differentiation was triggered with 5 $\cdot$ 10⁻⁷ M dexamethasone, 0.5 mM isobutylmethylxanthine and 50 μ M indomethacin. All inducers were obtained from Sigma-Aldrich. Medium was changed at three- to four-day intervals, and cultures maintained for 16 days.

2.3. Alkaline-phosphatase-activity measurements

Alkaline phosphatase, liver/bone/kidney (tissue-nonspecific; ALPL) activity was quantified in cell cultures induced to differentiate into osteoblasts. Cells were grown in P12 plates from Nalgene-Nunc, detached as described above and lysed

in the presence of phosphate-buffered saline (PBS) solution with 0.1% Triton, using a one-ml syringe plunger. A total of three to 20 μ l of resulting extract were mixed with 200 μ l solution, containing alkaline-phosphatase (AP) substrate (p-nitrophenyl phosphate) from Sigma-Aldrich. Reactions were incubated at 37 °C for 30 min and stopped with 50 μ l 3M NaOH. Generated products were quantified by 405 nm absorbance using GENios ELISA microplate reader from Tecan (Mannedorf, Zurich, Switzerland). Absorbance values were normalized with genomic DNA amounts in cell extracts. Relative DNA quantities were measured by quantitative real-time polymerase chain-reaction (QRT-PCR), with 18S ribosomal DNA (rDNA) primers (Table 2) and QuantiTect SYBR Green PCR Kit from Qiagen (Hilden, Germany), using Mx3005P qPCR System from Stratagene - Agilent Technologies (Santa Clara, CA USA).

2.4. Quantification of gene expression by QRT-PCR

Expression of osteoblastic-differentiation *RUNX2* and *ALPL* marker genes was analyzed by QRT-PCR at days six and 13 after induction. Likewise for adipocytic-differentiation *PPARG2* and lipoprotein lipase (*LPL*) marker genes. In short, total RNA was isolated from cells grown in P6 plates from Nalgene-Nunc, using TRI Reagent from Sigma-Aldrich and incubated with deoxyribonuclease I (DNase I) from the same manufacturer at 37 °C for 1 h to remove genomic DNA contamination. RNA was quantified by absorbance at

260 nm, using NanoDrop ND-1000 spectrophotometer from NanoDrop - Thermo Fisher Scientific. RNA integrity was checked by agarose-gel electrophoresis (AGE). Total RNA (1 µg) was retrotranscribed into cDNA using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA USA).

PCR amplification was carried out with 50 ng cDNA and 10 pmol of each primer, using QuantiTect SYBR Green PCR Kit in Mx3005P qPCR System. Cycle threshold (Ct) normalization was accomplished with 18S rDNA as constitutive gene, using about 0.6 ng cDNA. PCR profile included 95 °C for 15 min (DNA polymerase activation), and 45 amplification cycles [94 °C for 15 sec (DNA denaturation), 60 °C for 20 sec (primer annealing) and 72 °C for 20 sec (primer extension)]. Results were analyzed with MxPro QPCR version 3.00 software from Stratagene - Agilent Technologies. Analyzed genes and primers used are show in Table 2.

Table 2. Genes and primer sequences used for PCR amplification.

Gene	Primer sequence (5' → 3')	Product size (bp)
18S Ribosomal 5 (<i>RNA18S5</i>)	TACCTGGTTGATCCTGCCAGTAGCATATGCTTG TTAATGAGCCATTTCGCAGTTTCACTGTACCG	104
Alkaline phosphatase (<i>ALPL</i>)	CCAACGTGGCTAAGAATGTCATC TGGGCATTGGTGTGTACGTC	175
Runt-related transcription factor 2 (<i>RUNX2</i>)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Peroxisome proliferator-activated receptor gamma 2 (<i>PPARG2</i>)	GCGATTCCTTCACTGATACTG GAGTGGGAGTGGTCTTCCATTAC	136
Lipoprotein lipase (<i>LPL</i>)	AAGAAGCAGCAAAATGTACCTGAAG CCTGATTGGTATGGGTTTCACTC	113

2.5. Mineralized extracellular-matrix (osteoblasts) and fat droplet (adipocytes) staining

Extracellular-matrix mineralization of MSC induced to differentiate into osteoblasts was assessed with alizarin-red staining at day 16 after starting differentiation. In short, cultures in P12 plates were fixed with 3.7% formaldehyde for 10 min. Then, calcium deposits were stained with solution containing 10 ml of 1% alizarin red (w/v in distilled water) and 1 ml of 1% ammonium hydroxide (both chemicals from Sigma-Aldrich) for 45 min. Wells were then washed with distilled water, 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, as described elsewhere (Gregory et al., 2004).

On the other hand, fat-droplet accumulations inside adipocytes were determined by oil-red staining at day 16 after starting differentiation. Briefly, cultures in P12 plates were fixed with 3.7% formaldehyde for 20 min. Cells were washed with distilled water and stained with solution containing 8.2 ml of 0.3% oil red (w/v in isopropanol) and 6.8 ml of distilled water for 15 to 20 min. Then, wells were washed with distilled water, stained with hematoxylin and visualized with optical microscope. Staining assessments were carried out eluting stain with isopropanol at room temperature for 10 min, and further spectrophotometric-absorbance quantification of resulting eluates at 510 nm. Values were normalized, estimating total number of cells per well after crystal-violet staining. In short, once oil red was eluted, cells were stained with 0.1% crystal

violet in 10% ethanol for 20 min. Then, cells were washed six times with distilled water and stain eluted with 10% acetic acid for 20 min. Resulting eluate absorbances at 590 nm were quantified. Fat vesicles in cultures were expressed as absorbance ratio ($A_{510\text{ nm}}/A_{590\text{ nm}}$).

2.6. Statistical analyses

Experiments were carried out by triplicate, showing average $\pm$ standard error of the mean (SEM). Analysis of Variance (ANOVA) and “Fisher’s projected least-significant difference” (PLSD) tests were used to calculate p values. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. D6 intake effects on postmenopausal-women serum composition

D6 treatment for 60 days increased 8% and 18% serum levels of vitamin E and coenzyme Q10, respectively, with a statistically non-significant increment in 25 hydroxyvitamin D₃. No changes were observed for retinol and GSH serum-levels, whereas GPx was reduced near 12%. Besides, lipid peroxidation products were significantly reduced by 44%, and OxLDL was reduced more than 35.1% (Table 3).

Table 3. Variations in vitamins and antioxidant markers of postmenopausal-women serum after D6 intake.

Condition	LPO (nmol/ml)	GSH (nmol/ml)	GPx (activity units/ml)	PAO (μ mol/l)	OxLDL (μ g/ml)	25OHVit D ₃ (ng/ml)	Vit A (ng/ml)	Vit E (μ g/ml)	Q10 (ng/ml)
Pre-treatment	144	61.6	10.3	134	97	15.6	0.7	5.6	1.4
Post-treatment	80	63	9.1	132	63	17.5	0.7	6.06	1.65
Variation (%)	-44.4*	2.3	-11.7*	-1.5	-35.1*	12.2	0.0	8.2*	17.9*

* p<0.05.

3.2. Postmenopausal-women serum after D6 intake enhances MSC differentiation into osteoblasts

Postmenopausal-women serum at day six after starting differentiation of MSC into osteoblasts induced *RUNX2* gene in relation to medium supplemented with FBS. But such gene was less upregulated with both sera supplements than just FBS at day 13. Comparison of O-PreS and O-PosS showed that induction of this gene was higher when media was supplemented with sera from post-D6 treatment, being significant at day six. Additionally, *ALPL* gene was also upregulated by such sera supplements in relation to cultures maintained with just FBS at day six. As before, O-PosS significantly increased gene expression in relation to O-PreS at such time. Yet, no significant differences were observed among tested cultures at day 13 after starting differentiation (Fig. 1).

Figure 1. Expression of osteogenic genes. *RUNX2* and *ALPL* were analyzed in MSC induced to differentiate into osteoblasts at days six and 13. Culture media contained 10% fetal bovine-serum (O-FBS), 10% serum from pre-treated postmenopausal women (O-PreS) or 10% as before, but post-treatment (O-PosS). Gene-expression values are shown as number of times (fold) in relation to cultures uninduced into differentiation. * $p < 0.05$.

Alkaline phosphatase (ALPL) enzymatic activity showed a similar behavior than *ALPL*-gene regulation at both days, with higher ALPL activity for O-PosS cultures at day six, and no significant differences at day 13 (Fig. 2a).

Additionally, mineralization differences between O-PreS and O-PosS were non-significant, as revealed by alizarin-red staining (Fig. 2b). Yet, it was statistically significant for O-PosS when compared to O-FBS (Fig. 2c). Thus, results showed that postmenopausal-women serum after D6 treatment enhanced MSC osteoblastic differentiation.

Figure 2. Analyses of osteogenic markers. a) ALPL enzymatic activity at days six and 13 after MSC osteogenic induction in O-FBS, O-PreS or O-PosS media. ALPL-activity values were expressed as number of times (fold), in relation to

cultures uninduced into differentiation. **b)** Alizarin-red staining, corresponding to control uninduced cultures, or induced into osteoblastic differentiation, showing extracellular-matrix mineralization, in same media as “a”, but at day 16. **c)** Quantification of extracellular-matrix mineralization at day 16 for O-FBS, O-PreS and O-PosS cultures. * $p < 0.05$.

3.3. Postmenopausal-women sera after D6 intake have more capacity of inhibit MSC differentiation into adipocytes

A relevant inhibition of *PPARG2* and *LPL* adipogenic genes was observed for both A-PreS and A-PosS, in relation to A-FBS cultures, at days six and 13 after starting MSC differentiation into adipocytes. Even more, no *PPARG2*-gene expression was detected for A-PosS at both studied times, with *LPL* being barely detected at day 13. Thus, downregulation was higher with A-PosS than A-PreS, which showed some expression for both studied genes at both times (Fig. 3a). Additionally, lipid-droplet formation was lower in media supplemented with both sera, and mostly for A-PosS, with significant differences in relation to A-PreS (Fig. 3b).

Figure 3. Analyses of adipogenic genes and markers. MSC were induced to differentiate into adipocytes in medium containing 10% fetal bovine-serum (A-FBS) or 10% serum from postmenopausal women before (A-PreS) or after (A-PosS) D6 treatment. **a)** Expression of *PPARG2* and *LPL* adipogenic genes in MSC induced to differentiate into adipocytes, in different media at days six and 13. Gene-expression values are shown as number of times (fold) in relation to A-PreS expression at day six. ND: not-detected gene expression. **b)** Oil-red staining, corresponding to control uninduced cultures, or induced into adipocytic

differentiation, showing fat droplets, in same media as “a”, but at day 16. Lower plot shows staining quantification for such cultures. * $p < 0.05$.

4. DISCUSSION

Two-month intake of natural extract from orujo olive-oil refinement (D6) did not change retinol or vitamin D (measured as 25 hydroxyvitamin D₃) serum levels, the latter being deficient. Yet, it significantly increased coenzyme Q10 and vitamin E, which gives it a great potential as therapeutic antioxidant. Q10 is a lipophilic compound, initially characterized as component of mitochondrial respiratory-chain, where it functions as a coenzyme facilitating ATP generation, required for oxidative phosphorylation. Interestingly, much of recent therapeutic interest in Q10 is related to its property as the only known lipid-soluble antioxidant being endogenously synthesized (DiNicolantonio et al., 2015). It is well known that Q10 protects phospholipids and mitochondrial-membrane proteins from peroxidation, as well as DNA against oxidative damage that accompanies lipid peroxidation. Besides, recent data suggest that Q10 from circulating blood may influence intracellular targets, exerting protective effects against oxidative DNA-damage (Ozgocmen et al., 2007; Zhang et al., 2011). However, Q10 levels in blood decrease with aging, being associated to loss of antioxidant capacity (Niklowitz et al., 2016). Interestingly, vitamin E seems to be the only biologically-active metabolite in humans functioning as free-radical scavenger (Niki and Traber, 2012). Therefore, its increase together with that of

Q10 shows that D6 intake may enhance antioxidant capacity of serum in treated women, besides markedly downregulating *LPO* and *OxLDL*.

The high squalene-content in D6 distillate (>7%) can be responsible for its healthy effects. Indeed, it has been reported that squalene supplementation is cardioprotective in rats, due to its antioxidant properties (Farvin et al., 2007). Additionally, our results support the ones by others using animal models, showing that squalene-derived products increased coenzyme Q synthesis and levels, both in vitro and in vivo (Bentinger et al., 2014). Also, diet supplementation with squalene may improve liver mitochondrial-function, reducing oxidative stress effects with aging (Buddhan et al., 2007). These and our data suggest that D6 intake may protect against aging-associated pathologies, since oxidative stress has been proposed as a potential pathogenic mechanism linking aging and age-related pathologies (Davalli et al., 2016). Among them is osteoporosis, which may be prevented by Q10, as demonstrated in osteoporosis-induced rats by spinal-cord injury (Zhang et al., 2015). Additionally, in-vitro studies have demonstrated that Q10 reduced osteoclast formation from bone-marrow-derived monocytes (BMM) and RAW264.7 mouse cells. Besides, it enhanced osteoblastic differentiation in MC3T3-E1 cells, showing positive correlation between Q10 concentration used and mineralization level (Moon et al., 2013). These results suggest that Q10 increase due to D6 intake may prevent bone-mass loss associated to aging. Indeed,

reduction of antioxidative defenses in postmenopausal women has been associated to higher osteoporosis risk (Maggio et al., 2003).

Lipidic peroxidation showed higher levels both in plasma and erythrocytes in osteoporotic in relation to non-osteoporotic postmenopausal women (Ozgocmen et al., 2007). Also, lipidic peroxidation, quantified by thiobarbituric acid-reactive substances (TBARS), was also higher in both blood and saliva in a group of 22 postmenopausal osteoporotic women (also showing lower antioxidant-capacity) in relation to 22 control ones (Yousefzadeh et al., 2006). Indeed, our results showed that D6 intake reduced lipidic peroxidation, and thus may potentially protect against bone-mass loss. Yet, antioxidant capacity (AOC) did not change with D6 intake, as with GSH and GPx. Interestingly, this indicates that D6 did not increment antioxidant activity for studied molecules, but significantly reduced production of oxidized products, as shown by reduction of lipidic peroxidation and OxLDL. These results highlight the D6 intrinsic antioxidant-potential. Thus, the final D6 balance was a higher antioxidant-defense.

Additionally, increased OxLDL occurs with aging (Paik et al., 2013) and is a risk factor for multiple degenerative and age-related diseases (Gradinaru et al., 2015), like atherosclerosis (Mitra et al., 2011). Interestingly, epidemiologic studies have shown association between such disease and reduced bone mass (Farhat et al., 2007). This may happen by two mechanisms. Firstly, oxidized lipids may interact with bone via T lymphocytes, inducing receptor activator of

nuclear-factor kappa- β ligand (*RANKL*) gene expression, which enhances osteoclastogenesis (Graham et al., 2009). Secondly, such oxidized lipids may have a direct effect on osteoblasts. Thus, it has been reported that minimally modified (oxidized) low-density lipoproteins (Mm-LDL), as well as other products of lipidic peroxidation, may increase alkaline-phosphatase activity and mineralization on vascular cells, further inhibiting MC3T3-E1 preosteoblastic cellular-line differentiation into osteoblasts. Such effects were not observed with native (reduced) LDL (Parhami et al., 1997). Additionally, low and high OxLDL-concentrations increased osteoblast proliferation and apoptosis, respectively. This is in agreement with low osteoblast OxLDL-degradation rate, inhibiting differentiation of precursor cells into osteoblastic phenotype (Brodeur et al., 2008).

Therefore, D6 capacity to reduce OxLDL serum-levels may protect not only against cardiovascular diseases, but also against bone-mass loss. Actually, our results showed that serum from women after D6 intake had higher capacity to both activate osteoblastic, as well as inhibit adipocytic, differentiation from MSC than serum before such dietary supplement. That may be due to oxidized-lipid reduction after D6 intake. This is supported by results from others, showing that Mm-LDL (but not native LDL) inhibited M2-10B4 murine marrow stromal-cell differentiation into osteoblasts, further activating their differentiation into adipocytes. The latter effect was also observed with 3T3-L1 preadipocytes (Parhami et al., 1999). Besides, OxLDL (but not LDL) may induce *PPARG* gene

expression (Taketa et al., 2008), which is a transcription factor required to trigger adipogenesis. Other authors have also found that the presence of other oxidized lipids, like the ones derived from oxidized arachidonate-containing phospholipids (OxPAPC), may favor adipogenesis and inhibit osteoblastogenesis of MSC in vitro. Besides, such authors found higher OxPAPC concentrations in serum from osteoporotic versus normal women (Valenti et al., 2011). Indeed, lower oxidized-lipid concentrations in serum of women after D6 treatment may generate less-oxidant extracellular environments, which do not favor adipogenesis (Imhoff and Hansen, 2010). Besides, natural PPARG-ligands include oxidized fatty acids, like 9-(S)-hydroxyoctadecadienoic acid (9-HODE) and 13-(S)-hydroxyoctadecadienoic acid (13-HODE), being both present in oxidized LDL particles (Itoh et al., 2008).

Therefore, our results suggest that the adipogenic inhibitory effect on MSC observed when post-D6 serum was used may be due, in part, to both lower oxidizing and PPARG-agonist compounds after treatment. Additionally, the variations in serum composition may favor osteoblastogenesis. Although MSC from healthy premenopausal women has been used in the present study, we believe that similar positive results could have been obtained with MSC from postmenopausal women. MSC functionality and regenerative capacity are reduced with aging. That is mainly caused by variations in cellular niche. Such factors include increasing ROS and proinflammatory cytokines, favoring

senescence, apoptosis, DNA damage and mitochondrial dysfunction in stem cells (Oh et al., 2014). Indeed, reduction of estrogens favor ROS production and bone-mass loss in postmenopausal women (Almeida et al., 2007). That may explain why sera from such women favor adipogenesis in vitro, in relation to premenopausal ones (Stringer et al., 2007).

Yet, several authors have shown that MSC from persons of different age exhibit similar differentiation potency (Justesen et al., 2002), albeit with differences in gene expression (Wagner et al., 2009). Interestingly, it has been found that MSC from old rats increased proliferation and osteogenic differentiation, reducing the adipogenic one, when grown in presence of serum from young rats (Geißler et al., 2013). These results suggest that cellular microenvironments may play a key role, modulating both proliferation and differentiation capacities of cells. This is an interesting consideration for future investigations, to further analyze the effects of D6 intake on different aging aspects.

Our data also showed that FBS had lower and higher capacity to induce MSC differentiation into osteoblasts and adipocytes, respectively, than both pre- and post-D6 serum. This highlights the relevance of serum composition in MSC differentiation, which should be taken into account for both in vivo and in vitro studies, as also pointed out by other authors (Josh et al., 2012). In summary, our results show that intake of highly-antioxidant dietary supplements like D6 may modulate extracellular environments, favoring bone-mass formation and reducing adipogenesis. Thus, such extract could be potentially used as both

dietary supplement, as well as to design drugs to decrease aging effects. This could be useful for prevention and treatment of prevalent pathologies, like osteoporosis and obesity.

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CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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HIGHLIGHTS

- 1- D6 by-product of olive-pomace oil refining is rich in bioactive compounds.
- 2- D6 intake by postmenopausal women reduced lipidic peroxidation and oxidized LDL.
- 3- D6 intake by postmenopausal women increased vitamin E and CoQ10.
- 4- D6-treated-women serum favors osteoblastogenesis, inhibiting adipogenesis in MSC.
- 5- D6 intake may protect against aging and osteoporosis.