Induction of Osteogenic MC3T3-E1 Cell Differentiation by Nacre and Flesh Lipids of Tunisian Pinctada radiata

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Induction of Osteogenic MC3T3-E1 Cell Differentiationby Nacre and Flesh Lipids of Tunisian *Pinctada radiata*

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Abstract:

The flesh of the *Pinctada radiata* pearl oyster from coastal Tunisia is considered as a high source of n- 3 and n-6 and its shell nacre layer is a promising osteogenicbiomaterial. Fatty acid (FA) analysis showed that the major components found in total FA (TFA) were 14:0, 16:0, and 18:0 saturated FA (SFA); 16:1, 18:1, and 20:1 monoenoic FA; 20:4n-6 (ARA), 22:5n-3 (DPA). Characteristically high levels of 20:5n-3 (EPA) and 22:6n-3 (DHA) (6.53—89.75 mg/100 g TFA) polyunsaturated FA (PUFA) were found, respectively, in the TFA of nacre and flesh. Evaluatedthe effects *in vitro* of lipids extracted from nacre (Ln) and from flesh (Lc) of *P. radiata* on growth and the differentia- tion of osteoblasts. Cytotoxicity tests (3-(4, 5-dimethylthia- zolyl-2)-2, 5-diphenyltetrazolium bromide [MTT] and lacticacid dehydrogenase c [LDH]) demonstrated that both extracts are nontoxic. Alizarin Red staining was used in an osteoblast differentiation model using the osteoblast MC3T3-E1 cell line. It showed that the FA of both extracts induced osteoblast differentiation leading to mineralization. Reverse transcription-polymerase chain reaction (RT-PCR) showed a significantly higher expression of osteocalcin (*Bglap*) and runt-related transcription (*Runx2*) in MC3T3-E1cells in the presence of Ln. No difference of osteopontin (*Spp1*) and Collagen type I (*Col1a1*) genes compared to thecontrol was observed. In conclusion, these results supported, obtained from our *in vitro* experimental model used, the interest/potential of lipids extracted from nacre and *P. radiata* flesh to stimulate bone formation.

Introduction

The *Pinctada radiata* pearl oyster is found in coastal Tuni- sia, and is an Indo-Pacific origin sessile benthic species (Leach, 1814). Historical records first note its presence (Antit et al., 2011) as early as 1890 (Vassel 1897) in the gulf of Gabès (south of Tunisia), (Antit et al., 2011; Zakhama-Sraieb et al., 2009). However, following sporadic repatriation in the Gulf of Tunis, population numbers have fluctuated significantly (Tlig-Zouari et al., 2009), although, heavy metal contamination in these species is noted to be the lowest of four examined oyster species (Rabaouiet al., 2014). The study of Bellaaj-Zouari et al. (2012) revealed that the *P. radiata* pearl oyster exhibits considerable phenotypic plasticity related to differences in environmental and/or ecological conditions along Tunisian coasts, andhighlighted the discriminative character of the thickness of the shell's nacreous layer.

Nacre thickness of the collected *P. radiata* oysters range from 0.32 (La Marsa) to 2.07 mm (Maharès), with a mean value of 0.93 mm. This species represents a source of polyunsaturated fatty acid (PUFA) (especially n-3 (eicosapentaenoic acid [EPA] and doc- osahexaenoic acid [DHA]), particularly in phosphatidylcholine [PtdCho] and in phosphatidylethanolamine [PtdEtn] as well asn-6) (Ben Ammar, 2014; Ben Ammar et al., 2014).

We previously reported, for the first time, the fatty acid (FA) composition of nacre in the shell of this species (Ben Ammar, 2014) and because lipids are components of bone,expand our study to determine the impact of lipids from *P. radiata* on bone function. Kajarabille et al. (2013) reported on largely bound fat intake and bone articular pathologies. Some studies on humans indicate that long- chain poly unsaturated fatty acids (LCPUFA) can increase bone formation, affect peak bone mass in adolescents, and reduce bone loss (Kajarabille et al., 2013). The osteogenic potential of the nacre of mollusk shells is significantly documented (Atlan et al., 1997, 1999; Lamghari et al., 1999; Lopez et al., 1992; Silve et al., 1992). This suggests that it may be a perfect alternative natural biomaterial for bone regeneration as it shares properties that are found within bone itself (Checa and Rodriguez-Navarro, 2005). The similarity between the shell and the bone could be due to the presence of an organic component within the shell that plays a function similar to that of the bone organic matrix (Kikuchi et al., 2004).

New evidence supports the idea that dietary FA and anti-oxidants can reduce the osteoclast activity and reduce

the severity of osteolytic bone diseases and joints (Seifert and Watkins, 1997), but no studies have tested the effect of lipids in direct contact with the cells *in vitro*. However, many works on lipid bilayers *in vitro* or *in vivo* indicate that enrichment of PUFA, especially DHA, induces an increase in membrane permeability due to a decrease in the compactness of the membrane (Mitchell and Litman, 1998; Stillwell and Wassall, 2003). A review details the activities of lipids and PUFA on the bone biology and bone cell function (Watkins et al., 2001).

Lipids extracted from the nacre of the *Pinctada margaritifera* pearl oyster were tested on artificially dehydrated skin explants (Rousseau et al., 2006). However, the lipids of the nacre and the flesh of bivalve mollusks were never tested on osteoblast cells. This study constitutes the first test of *P. radiata* nacre and flesh lipids on osteoblastic cells.

MC3T3-E1 cells line, a pre-osteoblastic lineage deriving from newborn mouse calvarias (Rousseau et al., 2003; Wang et al., 2011), has been found to widely mimic osteo- blast behavior in the context of differentiation studies.

The aim of the present study is to examine the effect of nacre lipid extract (Ln) and the flesh lipid extract (Lc) of *P. radiata* on osteogenesis and mineralization in MC3T3-E1 cells. We used pre-osteogenic cells of murine MC3T3-E1 mouse, which were cultured in a monolayer. We used gene expression to assess differentiation in MC3T3-E1 cells. We also assessed the cytotoxicity of these lipids and impact on cell viability. We performed Alizarin Red staining to visualize the cell mineralization capacity.Materials and Methods

Animals

The measures of oyster samples were performed in sextu-plicates: Shell Length, 8.7 ± 0.57 (cm); Shell Width,
7.83 ± 0.42 (cm); and Total body (flesh and shell) Weight, 106.25 ± 7.26 (g).

Nacre Harvesting

The chosen animals were first weighed. Then, the shells were removed from their flesh, washed with water and ultrasound. After drying the shells in open air, the nacre was obtained *via* a mechanical extraction to obtain pieces after scraping the two most superficial layers with the aid of an electric micromotor equipped with a dental bur. The pieces of nacre obtained were first crushed using a mortar, then in a Retsch RM100 grinder to produce the nacre pow- der. The sieving of this powder was carried out by means of a Retsh type sieve (AS 200 basic) at an amplitude of 70–90 V for 40 min. Meshes ranging from 50 to 150 µm were used. The nacre of the inner shell layer of the *P. radiata* pearl oyster was ground into a fine powder to obtain particles of 50 µm (Ben Ammar, 2014).

Flesh Retrieval

The flesh was removed from the collected specimens of the Maharès area (Tunisia) (Ben Ammar et al., 2014). Samples were fixed in boiling water to inactivate enzymatic activity, especially phospholipases (Shewfelt, 1981), before being stored at −28 ^{0}C.

Lipid Extraction

Lipids were extracted according to the Folch et al. (1957) method with the solvent mixture chloroform–methanol (2:1, v/v) containing 0.01% butylatedhydroxytoluene (BHT) as an antioxidant (Christie, 1982).

Extraction of Lipids from Nacre (Ln)

We chose the finest particle size of 50 µm for a maximum extraction using 30 mL chloroform-methanol containing 0.01% BHT as an antioxidant per /g of nacre powder. After centrifugation, the lower phase was recovered. The extract was subsequently evaporated using a rotavapor and then dried under a continuous stream of nitrogen to prevent the oxidation of the FA and contact with air. The powder weight obtained was

recorded during this process.

Extraction of Lipids from Flesh (Lc)

We used whole animal to determine total lipid concentra- tion. The flesh (n = 6) was milled in a mixture of chloroform-methanol solvent (2:1, v/v) containing 0.01% BHT (as an antioxidant). After centrifugation, the lowerphase was recovered.

The extract was subsequently evaporated to dryness under a continuous stream of nitrogen. Samples were then concentrated to 10 mg/mL.

Fatty Acid Analysis

After evaporation to dryness, lipid extracts were trans-esterified according to the Cecchi et al. (1985). Methyl non-adecanoate 19:0 (Sigma-Aldrich Co. LLC, St. Louis, MO) was added as internal standard. Separation of fatty acid methyl ester (FAME) was carried out on a HP 6890 gas chromatograph with a split/splitless injector equipped with a flame ionization detector at 275 ^{0}C, and a 30 m HP Inno- wax capillary column with an internal diameter of 250 µm and a film thickness of 0.25 µm. Injector temperature was held at 250 ^{0}C.
The oven was programmed to rise from 50 to 180 ^{0}C at a rate of 4 ^{0}C/min, from 180 to 220 ^{0}C at 1.33 ^{0}C/min, and to stabilize at 220 ^{0}C for 7 min. Carrier gas was nitrogen.

FA peaks were integrated and analyzed using HP chemstation software.

Identification and Quantification of Fatty Acids

The different FA in *P. radiata* were obtained by comparing the retention times of the FA under study and those of mixture of methyl esters SUPELCO (PUFA-3).

The quantification of the FA was based on an internal standard not present in our samples, methyl nonadecanoate or 19:0 (Sigma). The two C22 dienoic acids, 22:2i and 22:2j, which are considered to be 22:2Δ7, 13 and 22:2Δ7,
15 (Gilles, 2009), respectively, were assigned in accor- dance with the data presented by Ackman (1986).

MC3T3 Cell Line

MC3T3-E1 cells, a clonal pre-osteoblastic cell line that stemmed from newborn mouse calvarias, were provided by the European Collection of Cell Cultures (Sigma Aldrich, 99072810). Cells were cultured in an α-Minimal Eagle Medium (αMEM, Gibco) supplemented with 10% FBS and penicillin/streptomycin antibiotics (1%, Gibco) and main- tained in a humidified atmosphere of 5% CO_2 at 37 ^{0}C with a medium change every 2 or 3 days until their use, as described in the literature (Luppen et al., 2003; Takeuchi et al., 1990).

Cell Culture

A desired amount of lipid extract was resuspended in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL before undergoing sterile filtration (0.22 µm pore size). Subsequently, it is divided evenly into several containers and stored at −20 ^{0}C.
Confluent MC3T3-E1 cells were trypsinized and plated in 24 well plates at a density of 5×10^4 cell/wellin 800 µL.
MC3T3-E1 cells were then treated for 14 days with the lipid extract of nacre (Ln) and flesh (Lc) at a similar concentration of 100 µg/mL and maintained in a complete 10% FBS (fetal bovine serum) (Dutscher), αMEM medium (Minimum Essential Medium 1X) (Gibco), (1% penicillin/ streptomycin antibiotics (100 U/ml/100 µg/ml, Gibco). The culture was supplemented with ascorbic acid (50 µg/mL, Sigma) and β-glycerophosphate (βGP) (10 mM, Sigma). Control wells were provided with the medium supplemen- ted with mineralization inductors. Medium was changed every 2 days. Cells were observed using light microscopy (Axiovert 25).

Cell Viability Assessment Via the MTT Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability. The MTT solution (Sigma, 5 mg/mL) was prepared by dis- solving MTT in phosphate buffered saline (PBS), after which it was filtered and sterilized. After 4 h of incubation at 37 ^{0}C, the MTT solution was discarded and 200 µL of the solubilization solution of DMSO (Sigma, USA) was added to each well plate to dissolve the formazan crystals while being incubated for 5 min at 37 ^{0}C. Aliquots of the resulting solutions were transferred to 96 well plates. The optical density of the solution in each well was measured at a wavelength of 550 nm using a Microplate Reader (Varioskan Flash Thermo Scientific).

Lactic Acid Dehydrogenase Cytotoxicity Test

The lactic acid dehydrogenase (LDH; Roche, ref 11–644–793-001) test was used to assess cell toxicity. LDH isa cytosolic enzyme present in many different cell types. Plasma membrane damage releases LDH into the cell culture medium. Extracellular LDH in the medium can be quantified by a coupled enzymatic reaction in which LDH catalyzes the con- version of lactate to pyruvate *via* NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490 nm.

The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity. Cultured cells were incubated24–48 h to induce cytotoxicity.

Seeding of wells, in triplicate, by nontreated cells consti- tuted the positive and negative control (respectively "high control" and "low control"). Medium filled in triplicate, in nonseeded wells, allowed the quantification of the back- ground noise of the culture medium. To achieve the "high control," cells were lyzed by the addition of 4 µL of Triton 100x solution, mixed thoroughly, and then transferred into an Eppendorf tube. This was then centrifuged at 1000 rpmfor 5 min and 100 µL of each cell supernatant for each con-dition was transferred to the 96 well plates and 100 µL of the detection reagent was added.

The plate was then incubated for 30 min at room temper- ature and protected from light. Finally, the plate was read on a microplate reader at 490 nm.

Alizarin Red Staining

To visualize cell mineralization capacity, Alizarin Red staining was performed according to Stanford et al. (1995).After 7, 10, and 14 days (n = 3), cells were fixed with 4% paraformaldehyde (Sigma), stained with Alizarin Red (1% w/v, Sigma) for 5 min, then washed with distilled water, and finally air-dried at room temperature. The stained cells were imaged using a LEICA microscope (M80).

The Alizarin red pigment determines the presence of cal- cium deposits by the cells thereby assessing the ability of these cells to mineralize. Mineralization is proportional to the red coloration.

To quantify the degree of staining, cultures were dis- tained with acetic acid (800 µL, 10% v/v) for 30 min under stirring at room temperature, as previously described (Gregory et al., 2004). The monolayer was scraped off, heated at 85 ^{0}C for 10 min, cooled with ice for 5 min, and centrifuged at 20,000 g for 15 min.

The resultant supernatant (500 µL) was finally removed and pH adjusted (pH 4.1–4.5) with ammonium hydroxide (200 µL, 10% v/v). Alizarin Red Staining concentrations, in each sample, were determined by absorbance measurement at 405 nm.

Real-Time Quantitative Polymerase Chain Reaction

RNA was extracted at different culture times and then con- verted into cDNA by RT for quantitative PCR. Total RNAfrom treated and untreated MC3T3-E1 cells were isolated using the RNeasy Kit (Qiagen). The first strand cDNA syn- thesis reaction was performed using 500 ng total RNA with a cDNA synthesis mix containing 200 U of MMLV Reverse Transcriptase (Gibco), 1RT buffer, 5 mM of dNTP, 200 mM of DTT, and 3 lg hexaprimers (100 pmol/µL). qPCR was carried out using SYBR Green qPCR Master Mix (Bio-Rad) and home-designed primers for glyceraldehyde-3-phosphate (*Gapdh*), and bone markers such as Osteocalcin (*Bglap*), Osteopontin (*Spp1*), and runt- related transcription (*Runx2*) and Collagen type I (*Col1a1*) (Eurogentec, Table 1) with the use of the StepOne system (Applied Biosystems) and the results were treated with the StepOne software.

These primers were specifically chosen and are presented. Cycling parameters were 15 min at 95 ^{0}C (initial denaturation); 35 cycles of 10 s at 95 ^{0}C (denatur- ation), 20 s at a specific temperature for each primer (hybridization), and 15 s at 72 ^{0}C (elongation). Gene expression was determined in three separate experiments and normalized using *Gapdh* then compared against a control gene.
Analyses and fold differences were determined using the comparative C^T method. Fold change was calculated from the ΔΔCT values with the formula $2^{\Delta\Delta C^T}$ and data were relative to control values at day 1 (Livak and Schmittgen, 2001).

Statistical Analyses

All the experiments which refers to lipid Composition were performed in replicate (n=6). Mean values and standard deviations were calculated. Data was analyzed using Statistica Version 6.0 to assess significant differences between means according to the ANOVA method. For this, the Duncan test was applied and differences were considered significant when $p<0.05$. This analysis was performed by XLSTAT software version 7.5.2. All other experiments were performed in triplicate. The results were expressed as the mean SD. All analyses were conducted using the SPSS version 14.0 software (SPSS, Chicago, IL).

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Results

Lipid Composition

The lipid composition of the nacre and flesh of *P. radiata* pearl oyster is presented in Table 2 with corresponding TFA. The TFA were slightly higher during winter, which coincides with the reproductive season of *P. radiata*.

The lipid extraction yield per oyster was 3.76% for flesh and 0.82% for nacre. The results of FA analysis showed the presence of 25 FA in the nacre powder and in the flesh, and revealed that the content of the TFA was, respectively, 87.97 and 400.21 mg/100 g TFA.
The most copious SFA in nacre and flesh were as follows: 14:0 (2.66–6.05%), 16:0 (25.70–34.00%), and 18:0 (34.02–12.61%) and monounsaturated fatty acid (MUFA): 16:1 (2.02–3.60%), 18:1 (5.12–2.80%), and 20:1 (0.63–1.40%). The most abundant PUFA were: 20:5n-3 (0.60–5.80%), 22:6n-3 (7.00–16.00%), and 18:4n-3 (0.50–1.00%). (n-6) PUFA contained higher percentages of 20:4n-6 than 18:2n-6. The percentage of 20:4n-6 in (n-3) PUFA was significantly higher than that in (n-6) PUFA ($p < 0.05$) (Table 2). We noticed also the presence of noninterrupted methylene dienoic (NMID) (22:2): 22:2i (0.30 and 0.63%) and 22:2j (0.26 and 0.33%) in small quantities.

LDH and MTT Test

Cell viability was directly monitored by the MTT assay at each dose point (Fig. 1b). Following the first dose of 100 µg/mL, viability with Lc increased slightly and no significant differences were noted between the two lipid extract type. Higher cell death was detected between con- centrations of 500 and 1000 µg/mL of the Lc and Ln extracts. The cell mortality of the Ln was significantly higher compared to the Lc from dose of 500 µg/mL. No significant difference was observed between the Lc and the Ln throughout the entire evaluation dose.

LDH tests demonstrated a steady increase in the response to cytotoxicity as the dose of lipid used increased, with higher cytotoxicity noted following the use of the lipids of nacre (Ln) compared to the lipids of the flesh (Lc) (Fig. 1a) These results correlate with those of the MTT assay demon- strating a decrease in cell viability following treatment with the lipids.

Lipid cytotoxicity significantly increased with the increasing concentration of Ln, from 100% to 200 µg/mL ($p < 0.01$) and 150% at 500 µg/mL ($p < 0.001$) into 250% to 1000 µg/mL ($p < 0.001$), but also less with Lc. It should be noted that in similar circumstances, there is a dose effect and a greater cytotoxicity of Ln. For the con- tinuation of the study, we choose to work with the dose of 100 µg/mL.

Lipid Extract Activity

Alizarin Red staining was performed to visualize the cell mineralization capacity. Control shows noactivity of mineralization at D7 and D10.
Over time, there is much more important deposition of calcium points until the 14th day of culture, and we can observe that mineralization is intense under Ln and Lc conditions.

From the 14th day of culture, the intensity of alizarin redcolor appears similar to Ln. The intensity of Alizarin Red staining color on MC3T3-E1 cells allowed us to infer that the pre-osteoblast MC3T3-E1 mineralizes ECM under the effect of both extracts from the 10th day onwards. In contrast, the mineralization starts from the D7 for Ln rel-ative to Lc ($p < 0.05$). Lipids of nacre significantly induce mineralization of cells from D14.
The qPCR was conducted to identify the effect of the two extracts on the variation in the gene expression involved in the ossification processes. We investigated the role of lipids in the induction of osteogenic transcription factors such as Runx2 that is activated at the beginning of osteogenesis and is essential for the differentiation of osteo- blasts. The analyses of gene expression were carried out on the MC3T3-E1 cells cultured in a monolayer, in a D1, D7,D10, and D14 series, in α MEM medium and in the presence of different extracts Ln and Lc with ascorbic acid and βGP.
All points are comparable with each other because they were all normalized to the control condition at D1 allowingthe observation of the evolution of the osteoblastic pheno- type over time.
Expression of *Spp1* is inhibited from the first to the 7th day of culture. An increase is observable on D14for the control and in the presence of the two extracts.Both extracts follow the same trend as the control unit. Comparing the expression of *Bglap* of the two extracts, it can be observed that there is a marked increase of *Bglap* expression in those cells exposed to Ln, whichis significantly higher than Lc at day 7. There is a similar expression level to the control on D10 and D14.

With regard to extract Ln, expression levels of *Col1a1*indicate that the cells enter into a differentiation phase at D10. It may be that Ln stimulates an input phase differentiation at D10. Indeed, mineral deposits are already obsery able at D7.
The expression of the gene *Runx2* is significantly increased from day 10 following treatment with Ln compared to control and Lc conditions. Ln induces a rapid increase in the expression of *Runx2*, visible from the first day of treatment and persisting after 10 days. Reduction of*Runx2* expression is observed in the presence of Lc until D7, followed by an increase in the *Runx2* expression after 10 days.

Discussion

Lipid Composition

In our Mediterranean species, the content of PUFA is high, which is in agreement with the literature (Ben Ammaret al., 2014).
According to Freites et al. (2002), these FA are probably related to several parameters, including the environmental abundance of organic detritus, which is an important source of 14:0, 15:0, 16:0, and 18:0. The dominant FA identified in nacre and shell were: 14:0, 16:0, 18:0, 16:1, 18:1, 18:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3 (Table 2). These FA contribute approximately 60–75% of the TFA.
The high amount of 16:0 in nacre seems to be important.Assessing the potentially stimulating or inhibitory effect ofvarious FA on the production of osteoclasts and osteo-blasts, it was determined that mainly the SFA of 14–18 chain length that inhibited the formation of osteoclasts, while they stimulated the growth and proliferation of osteo- blasts (Gille, 2011).

The most characteristic PUFA of *P. radiata* were found tobe DHA and EPA, which ranged 7.55-21.77% of the TFA in nacre and flesh, respectively. This finding characterizes the high nutritional value of this species (Ben Ammar et al., 2014). In corals of certain PUFA from marine nutrient sources supports coral growth (Papina et al., 2003). As a result, these PUFA participate well in the growth of the *P. radiata* species and particularly its nacre layer.
Also, we noticed the presence of NMID (22:2): 22:2i (from 0.30 to 0.63% of the TFA) and 22:2j (from 0.26 to 0.33 % of the TFA). According to several authors, the role of 22:2 NMID is unknown, but the association with mem- brane lipids and the selective retention in starved animals suggest a structural/metabolic function and/or a strong resistance against degradation (Boussoufa et al., 2011; Klingensmith, 1982). These FA (22:2i and 22:2j) areinvolved in the mechanisms of fluidity, integrity, and

structure of the membrane (Abad et al., 1995; Gilles, 2009).
According to Jeong et al. (1990) and Soudant et al. (1999), 22:2i and 22:2j can substitute for some essential fatty acids: 20:5n3, 22:6n3, and also 20:4n6. Mollusks have generally active FA elongation and desaturation systems permitting the *de novo* synthesis of NMID FA. These are the only PUFA that are synthesized by marine mollusks (Abad et al., 1995; Ojea et al., 2004).

Zhukova (1991) proposed that the biosynthesis of these FA occurs *via* the n-7 MUFA pathway until 20:1n- 7, which is desaturated by Δ5 desaturase to 20:2n-5 and then elongated to 22:2n-7. There is a possibility of facinga deficiency of dietary UFA, including the n-3 and n-6 PUFA. In fact, Ojea et al. (2004) reported that 22:2i and 22:2j in the polar lipids of *R.decussatus* were negatively correlated with EPA 22:2j and were also negatively correlated with DHA. In another study, Klingensmith (1982) found an inverse relationship between n-3 PUFA, especially EPA and DHA, and NMID FA in the clam *Mercenaria mercenaria* (Linnaeus 1758). Similarly, in previous work (Ben Ammar et al., 2014), the increase in C22:2i and C22:2j coincides with low levels of EPA and DHA, respectively.
The marine invertebrates, particularly bivalve mollusks, have a proven ability to synthesize *de novo* unique nonmethylene-interrupted (NMI) FA, such as 20:2 NMID and 22:2 NMID (Barnathan, 2009; Dridi et al., 2017).

LDH and MTT Test

The results clearly show a specific effect of Ln on cell death, but with a concentration beyond 500 mg/mL. Such an effect may partly be due to the conversion of the FA of the n-3 or n-6 for example. The conversion of arachidonic acid (ARA) to eicosanoids by COX enzymes (cyclooxy- genase) and LOX (lipoxygenase) leads to the production of superoxide anions and free radicals that contribute to the onset of oxidative stress (Samuelsson, 1987). Eicosanoids themselves could be involved in the observed apoptogen effect and this could be due either to the extraction of Ln, or due to sterilization. In addition, these FA (including n-3 EPA and n-6 GLA) exhibit antitumor or cytotoxic activity on carcinoma cells (Kuratko and Becker, 1998; Wigmoreet al., 1996).
Indeed, phospholipids play various roles in cellular metabolism, especially because of their participation in the composition of the cell membrane. Indeed, nacre was able to increase the cell osteogenic activity without any apparent toxicity (Green et al., 2015; Lopez et al., 1992). However, the dose used in our work for cell assay is compatible withlow toxicity and shows that the method of extraction of total lipids was able to eliminate all trace of organic solvent.

Lipid Extract Activity

The testing of Lc and Ln on MC3T3-E1 clearly demonstrates their effectiveness in modulating bone mineraliza- tion and potentially bone deposition. The results showed that *Spp1* is expressed at D14. *Spp1* has been described as an important molecule involved in bone remodeling (Duvall et al., 2007; Rittling and Matsumoto, 1998), it plays a role in cell adhesion and migration (Pampena et al., 2004) and in the mineralization of the extracellular matrix that produced *in vitro* cell cultures (Speer et al., 2005).
More specifically, the mineralization step results from the synthesis of *Bglap* in combination with other matrix proteins. However, only the Ln induces the mineralization of pre-osteoblasts and at the same time induces significant overexpression of *Bglap*.
The latter is one of the few markers of the differentiation of the cells into osteoblasts. Indeed, it is only expressed by differentiated osteoblasts (Kasugai et al., 1991) during the mineralization phase (Owen et al., 1990). It has been demonstrated that mice in which the gene encoding *Bglap* have a progressive increase in bone mass, and bone mineral content higher than wild-type mice (Boskey et al., 1998; Ducy et al., 1996; Luo et al., 1997). *Bglap* may act on bone formation by controlling the activity of osteoblasts and as an inhibitor of mineralization (Hunter et al., 1996). In addition, the expression of the *Bglap* gene shows an increase at day 7 following treatment with Ln and it is stable at D14 compared to the control. The difficulty of quantifying this marker hasalready been seen in other studies (Pereira et al., 2003).

The expressions of *Col1a1* are quite consistent with osteoblast differentiation (Owen et al., 1990). *Runx2* is the master gene for osteoblast differentiation, which is activated at the beginning of osteogenesis and is essential for osteoblast differentiation (Komori, 2010; Nakashima et al., 2002) regarding the expression and activation

of Bglap (Ducy et al., 1997). The expression of the *Runx2* gene is increased from D10 for Ln compared to the control and Lc conditions. These different extracts in this study induce a complete stimulation of these cells to the stage of cells that mineralize.

Ln and Lc extracts stimulated the increased production of markers associated with osteoblast differentiation compared with standard inducers. This was also found for the mineralized deposits that appear as early as at the 7th day for MC3T3-E1 cells compared to the control. In the literature, the stem cell deposits are normally observed after 18 days (Hanada et al., 2003) while MC3T3-E1 cells require 21 days (Sudo et al., 1983). Mineralized deposits are observed after 7 days of culture in the presence of lipids of nacre.

This is a considerable acceleration of the mineralization process, as the MC3T3-E1 cells normally mineralize after 3 weeks (in the presence of inducers) (Sudo et al., 1983). After 14 days of treatment with Ethanol Soluble Matrix (ESM) 100 µg/mL, Alizarin Red staining demonstrated the presence of precipitated calcium in osteoblasts and MC3T3-E1 cells (Brion et al., 2015). Indeed, in the presence of Ln and Lc, MC3T3-E1 mineralize depending on the duration of incubation.

Thus, using various tests, we have demonstrated the stimulation of osteogenesis by flesh and nacre lipids of *P. radiata* by using MC3T3-E1 pre-osteoblasts in the process of ossification. These results are similar to those of Moutahir-Belgasmi et al. (2001), which showed that the molecules extracted from nacre are recognized by cells of the skeleton. With these types of cells, Ln and Lc induce the activation of protein markers or specific genes of bone tissue.

In the present study, using only lipid extract of nacre and flesh of *P. radiata*, we found that these extracts are closely involved in pre-osteoblast differentiation by inducing engagement of MC3T3-E1 osteoblast lineage cells in the activation of the promoters of specific genes of bone tissue, such as *Col1a1*, *Bglap*, *Spp1*, and *Runx2*. Furthermore, overexpression of *Bglap* and *Runx2* by Ln compared to the control induced pre-osteoblast differentiation in pre- osteoblast MC3T3-E1 cells. Taken together, these results suggest that these lipid extracts stimulated osteoblast differentiation as an important part of the regulatory machinery involved in early osteogenesis. Recently, Wauquier et al., 2015 found at the single cell level that, one FA is able to trigger several different independent pathways all of which may play a role in the final cellular metabolic response.

These findings support our strongly research, namely that the FA of the nacre and the flesh can play an important role bone remodeling in humans suffering from various bone pathologies. We may find solutions to fight against aging and diseases of the bone. The marine environment is a valuable source of bioactive lipids and lipid extracts of nacre and flesh of *P. radiata*. Consistent and repeatable beneficial effects of n-3 FA on bone metabolism and bone. Watkins et al., (2001) showed that (PUFA) modulate eicosanoid bio-synthesis in numerous tissues and cell types, alter signal transduction, and influence gene expression.

Wauquier et al. (2013) showed that the lipid receptor GPR40 was found on the membrane is ablated of bone cells involved in bone remodeling. When the GPR40 receptor of these cells is stimulated, the cell differentiation of osteo- clasts is inhibited. Therefore, the bone is less absorbed, which curbs bone loss. The specificities of the individual FA composition may be at the origin of the integration of lipid extracts of nacre and flesh of *P. radiata* in cells.

As mentioned by Kim et al. (2012), nacreous agents pre-pared from the pearl oyster *Pteria martensii* prevent osteo- porotic bone loss associated with estrogen deficiency in mice mainly through osteoclast inactivation. PUFA seem to be modifiable risk factors for osteoporosis, but supplementation with n-3 PUFA is effective in osteoporosis (Weiss et al., 2005).

In conclusion, the induction of mineralization of MC3T3-E1 cells by nacre and flesh lipids of *P. radiata* was demonstrated here. However, if the current use of the spe- cies *P. radiata* is still the subject of controversy, our work pays attention to the quality of the *P. radiata* pearl oyster and the importance of the extraction process of the active ingredients. Combined with its nontoxicity and rapid metabolizable ability, lipid extracts can become an effective agent against osteoporosis. Ln and Lc could be used also for bone tissue repair due to the favorable properties. Any reasonable medicinal or industrial use should consider different variations for better development and use of the species.

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