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Mineralization by mesenchymal stromal cells is variously modulated depending on commercial platelet lysate preparations

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Abstract

Background aims. Numerous cellular models have been developed to investigate calcification for regenerative medicine applications and for the identification of therapeutic targets in various complications associated with age-related diseases. However, results have often been contradictory due to specific culture conditions, cell type ontogeny and aging status. Human platelet lysate (hPL) has been recently investigated as valuable alternative to fetal bovine serum (FBS) in cell culture and bone regeneration. A parallel comparison of how all these multiple factors may converge to influence mineralization has yet to be reported. *Methods.* To compare mineralization of human mesenchymal cell types known to differ in extracellular matrix calcification potency, bone marrow–derived mesenchymal stromal cells and dermal fibroblasts from neonatal and adult donors, at both low and high passages, were investigated in an *ex vivo* experimental model by supplementing the osteogenic induction medium with FBS or with hPL. Four commercial hPL preparations were profiled by liquid chromatography/ electrospray ionization quadrupole time-of-flight spectrometry, and mineralization was visualized by von Kossa staining and quantified by morphometric evaluations after 9, 14 and 21 days of culture. *Results.* Data demonstrate that (i) commercial hPL preparations differ according to mass spectra profiles, (ii) hPL variously influences mineral deposition depending on cell line and possibly on platelet product preparation methods, (iii) donor age modifies mineral deposition in the presence of the same hPL and (iv) reduced *in vitro* proliferative capacity affects osteogenic induction and response to hPL. *Conclusion.* Despite the standardized procedures applied to obtain commercial hPL, this study highlights the divergent effects of different preparations and emphasizes the importance of cellular ontology, donor age and cell proliferative capacity to optimize the osteogenic induction capabilities of mesenchymal stromal cells and design more effective cell-based therapeutic protocols.

Key Words: aging, bone marrow stromal cells, calcification, fibroblasts, platelets, regenerative medicine

Mineralization is a physiological process in hard connective tissues but can also be considered pathological when occurring in soft connective tissues. Although extensively characterized in *ex vivo*–expanded populations of mesenchymal stromal cells (MSCs), many facets related to osteogenic induction remain elusive [1,2]. Despite the ability to differentiate into calcifying osteoblasts *in vivo*, MSCs fail to differentiate and mineralize the extracellular matrix (ECM) in standard medium supplemented with fetal bovine serum (FBS). Therefore, to induce a temporal cascade of maturational stages toward the osteoblast phenotype that allows ECM mineralization [3], cells must be cultured in osteogenic induction medium (OIM, which contains β-glycerophosphate, ascorbic acid and dexamethasone) [4]. More recently, because platelets contribute to mineralization in both physiological and pathological microenvironments, influencing sites for mineral nucleation [5] and providing growth factors [6] as well as exosomal mediators [7], it has been shown that platelet-rich plasma can efficiently promote healing of hard and soft connective tissues, improving, for instance, bone regeneration in clinical trails [8]. Therefore, the fractured platelet derivative human platelet lysate (hPL) is increasingly favored as an FBS replacement for regenerative medicine clinical applications [9]. However, hPL sources and platelet product preparation methods can influence platelet number,

growth factor concentration and, consequently, osteogenic induction/differentiation outcomes [10–12]. Moreover, because individual hPL effectiveness can differ according to donor-specific variability (i.e., age, gender), anticoagulant (i.e., heparin) and storage [11,13], commercial platelet lysates derived from pooled donors are frequently used to improve consistency and lower batch variability [14,15]; nevertheless, divergent results may be observed.

Therefore, we have investigated the effect of different commercial platelet lysates on *ex vivo* mineralization comparing various mesenchymal cell types (human bone marrow [hBM]-MSCs versus human dermal fibroblasts), each with contrasting ECM mineralization propensities. In particular, human MSCs are multipotent progenitors with enormous potential for repair and regeneration of bone and cartilage. Although these cells can be isolated from a variety of tissues, those from bone marrow, being the most widely investigated and characterized [16], were used in the present study as representative of cells positively associated with efficient mineralization. Conversely, human dermal fibroblasts (HDFs) are mesenchymal cells derived from tissues that typically mineralize in pathologic conditions [17].

Moreover, calcification can be also influenced by functional changes related to donor age or reduced cell proliferation capability. For instance, replicative senescence in hBM-MSC cultures can impair bone progenitor osteogenic differentiation and, consequently, matrix mineralization [18]. Yet replicative senescence can enhance ECM calcification in soft connective tissue mesenchymal cells (i.e., smooth muscle cells, fibroblasts) by increasing the response to pro-mineralization stimuli. By contrast, studies on HDFs isolated from neonatal donors demonstrated that these cells are significantly less responsive to pro-osteogenic factors [19]. Higher incidence of mineralization-associated diseases, including either osteoporotic paucity of bone mineralization or aberrant calcification in soft connective tissues and atherosclerotic vasculature [20], is consistently associated with age.

The aim of this study was to evaluate whether (i) different commercial hPLs have the same influence on mineral deposition in different cell lines, (ii) reduced *in vitro* proliferative capacity affects mineral deposition upon hPL supplementation and (iii) donor age modifies mineral deposition in the presence of the same hPL.

Methods

Liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry analysis

Electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) accurate mass spectrometer (G6520A, Agilent Technologies), controlled by MassHunter (v. B.04.00) and interfaced with an HPLC-Chip Cube to an Agilent 1200 nano-pump was used for analysis.

Chromatographic separation was performed on an integrated HPLC-Chip (Agilent Technologies) with a 75-μm ID, 43 mm, 300 Å C18 column, before a desalting step through a 40-nL trap column. The injected sample (1 μL) was loaded onto the trap column with a 4 μL/min 0.1% FA:ACN (98:2) phase flow, and after 3 min, the pre-column was switched inline with the nanoflow pump (400 nL/min, phase A: water:ACN:FA 96.9:3:0.1, phase B: ACN:water:FA 94.5:5:0.1), equilibrated in 10% mobile phase B. Proteins were eluted from the reverse phase column through the following gradient: 10–90% mobile phase B for 5 min, held in 90% mobile phase B for 5 min and switched back to 10% mobile phase B for 3 min, for a total runtime of 40 min, including a 10-min post-run reconditioning step.

Mass spectra were recorded from 350 to 3200 m/z at scan rates of 1 Hz; the detector was operated at 2 GHz in extended dynamic range mode. Mass spectra were automatically recalibrated with two reference mass ions. Spectra were displayed and processed by the software MassHunter Qualitative Analysis (B05.00, Agilent Technologies).

Mass spectra across the whole chromatogram (0–26 min) were averaged and, after subtracting the background (obtained by averaging the mass spectra at the end of the run), the spectrum was deconvoluted by using the maximum entropy algorithm in the range of 10 000–150 000 Da.

Cell culture

hBM-MSCs were harvested from a 42-year-old male donor after informed consent, according to the Declaration of Helsinki and local ethical committee–approved procedures for isolation and immunephenotypic characterization, as previously described [16,21]. Briefly, cells were routinely grown in α-minimum essential medium (MEM) without nucleosides (Gibco Invitrogen), supplemented with 8% hPL (obtained from pooled batches of 50 donors), 1% L-glutamine (Gibco Invitrogen), 1 UI/mL heparin (Sigma-Aldrich), and 10 mg/mL ciprofloxacin (HIKMA) [16]. In this study, hBM-MSCs were selected among those obtained from six independent donors as representative of the behavior of hBM-MSCs. HDFs from adult tissue (aHDFs; Thermo Fisher Scientific, cat. # C-013-5C). To evaluate whether hPL can reverse the "resistant phenotype" of cells considered to be "low responders" to pro-osteogenic stimuli, we also used HDFs derived from neonatal tissue (nHDFs; Thermo Fisher Scientific, cat. # C-004-5C) [19].

HDFs were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco-Thermo Fisher Scientific) according to standard procedures [22].

Cell expansion

Cell expansion represents a preliminary step necessary to obtain the required number of cells at different passages (i.e., low and high) to be used for the mineralization assay. Cells (hBM-MSCs, nHDFs and aHDFs) were serially passaged in T25 flasks in their growth medium as specified in the previous paragraph. The number of population doublings (PD) for each cell type was determined. In particular, cell number was measured by hemocytometer, and PD was calculated using the following formula [23]:

PD = Ln (number of cells harvested)

     − Ln (number of cells seeded)/ln 2.

Cells at low passage (p3) were used when the number of PD was ≈3 ± 1 in all cell lines. Cells at high passages were used when hBM-MSCs, nHDFs and aHDFs were at p10, p40 and p30, respectively, and PD was ≈1 ± 0.2. Cell cultures were monitored using an inverted phase contrast microscope (Leica DM-IL).

Ex vivo mineralization assay

For mineralization experiments, cells seeded in 24-well plates at a density of 40×10^3 cells/well (DB Falcon), were treated with an OIM containing DMEM supplemented with ascorbic acid (50 μg/mL; Sigma), β-glycerophosphate (10 mmol/L) (Sigma) and dexamethasone (10 nmol/L; Sigma) and 10% FBS [3,24,25] or with FBS replaced by 8% or 5% commercial hPL, depending on the manufacturer's recommendation. Change from FBS- to hPL-containing OIM was well tolerated without cell toxicity. Because hPL and pro-mineralization reagents present in the OIM may influence proliferation rate [26,27], in a preliminary experiment, we tested various commercial hPLs on hBM-MSCs during the expansion phase. After 6 days of culture, cell number was 185 000, 273 000, 228 000 and 301 000 cells, depending on the commercial hPL used. Therefore, to allow a more uniform comparison, mineralization assessment was performed on cells expanded in their medium until confluence, as previously described.

As hPL sources, we used the following: two Stemulate liquid formulas produced by Cook Medical, one preparation requiring heparin (hPL1) and the other heparin-free (hPL2); a Good Manufacturing Practice–grade hPL liquid formula produced by Macopharma requiring the addition of heparin (hPL3); and a lyophilized formula, Lyset, produced by Sclavo

Diagnostics International, which is supplemented with anticoagulant by the manufacturer (hPL4).

Mineralization can be assessed by alizarin red (AR) or by von Kossa (VK) staining. Although AR is accepted as indicative of the presence of calcium phosphate deposits, it is a nonspecific stain for acid insoluble divalent complexes and does not specifically stain calcium-containing minerals or calcium itself [28,29]. Therefore, mineralization was assessed by VK, a precipitation reaction in which silver ions react with phosphate and, under ultraviolet light, silver is deposited to replace the reduced calcium of calcium phosphate [30]. Briefly, after 9, 14 and 21 days in OIM, cells were fixed in 4% paraformaldehyde, stained with 2.5% silver nitrate, placed under a ultraviolet lamp for 30 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for 2 min. VK-positive (dark) deposits were observed after alcohol washes.

Areas of mineralization were quantified on digital images by ImageJ software. Triplicate experiments were performed twice.

Statistical analysis

Statistical comparison was made using GraphPad software, version 6.0, and P values < 0.05 were considered significant. Statistical significance between treatments and time of culture in the same cell line was determined using two-way analysis of variance followed by Tukey's multiple comparison test. Independent t-tests were used to compare cells at low and high passages with the same time in culture and with the same OIM. Values are shown as mean values ± SEM.

Results

Mass spectra indicated differences between hPL commercial sources

The complex protein mixtures of four hPLs were analyzed by ESI-Q-TOF without gel separation and digestion. For all samples, a broad range of peaks showing intensities <0.5 across the selected 10 000 to 150 000 m/z range and a prominent base peak of intensity >1.5 between m/z ratios 66 440 to 66 557 (Figure 1). Interestingly, there was notable heterogeneity in number and peak intensities among the hPLs. For instance, hPL3 and hPL4 exhibited a peak at 76 102 and at 109 559 m/z, whereas hPL1 and hPL2 did not (Figure 1).

Effect of different commercial hPL on hBM-MSC-dependent mineralization

In line with expectations from previous studies [16], treatment of hBM-MSCs with OIM led to a
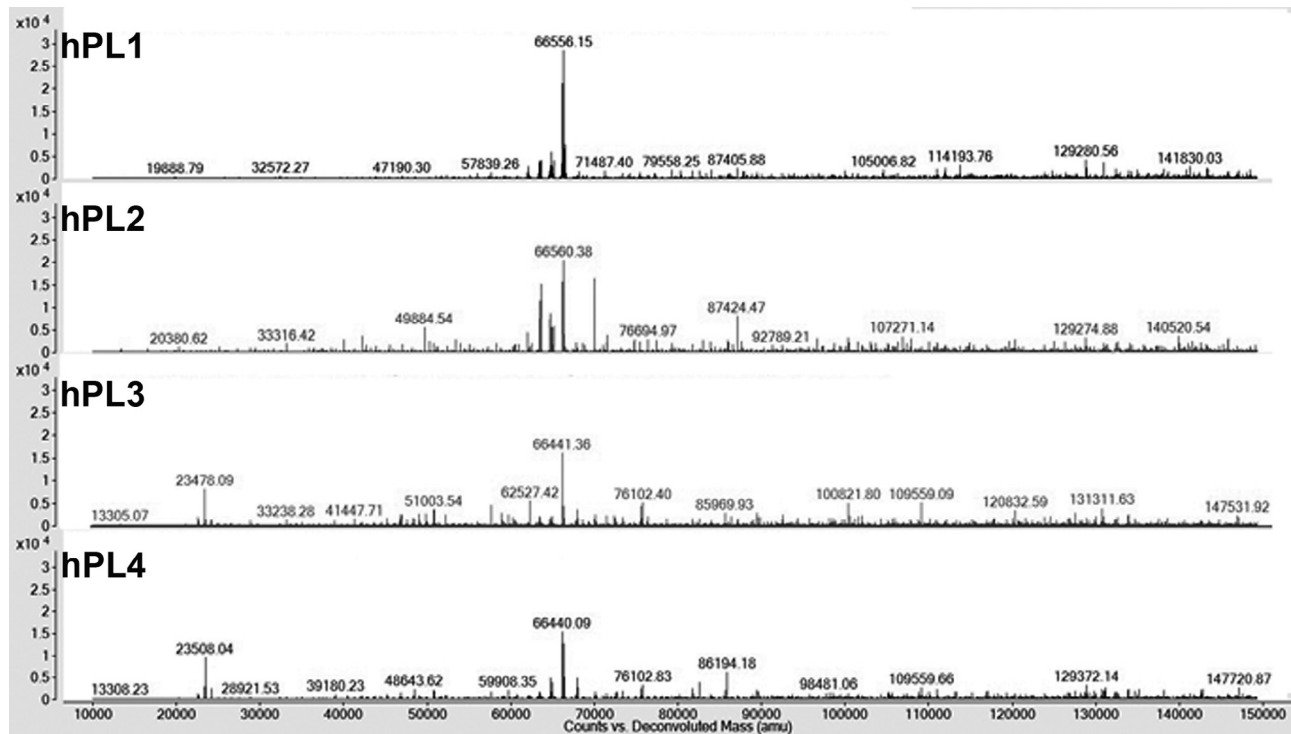
Figure 1. Liquid chromatography (LC)/ESI-Q-TOF profiling. Representative LC-mass spectrometry spectra of m/z intensities for peptides ranging from 10 to 150 kDa from four commercial hPL preparations. Each hPL was characterized by a specific peak profile indicating composition differences.

progressive increase of VK staining from 9 to 21 days. For OIM supplemented with hPL3 or hPL4, VK staining was >40% of the cellular monolayer at 14 days and >90% at 21 days (Figure 2). In the same cells, when OIM was supplemented with FBS, hPL1 or hPL2, VK staining was significantly ($P < 0.001$) reduced at 14 days compared with other hPL treatments. A similar trend was observed also at 21 days, when only 20% of the cellular monolayer appeared stained (Figure 2).

Effect of different commercial hPL on HDF-dependent mineralization

The overall pattern of calcification evidenced by VK staining of HDF cultures (Figure 2) was very different compared with that seen in hBM-MSCs. In particular, in aHDF cultures, VK staining at 9 and 14 days was similar for OIM supplemented with either FBS or any of the hPL sources (Figure 2). At 21 days, the only evident staining was in the presence of FBS and hPL1, although it never exceed 20% of the monolayer area.

In nHDFs, the use of FBS or hPL4 did not result in any appreciable calcification because VK staining was observed only at 21 days using hPL1, hPL2 and hPL3, although hPL3 demonstrated more than twice the extent of VK staining as that seen using hPL1 or hPL2.

Notably, in the same experimental condition (e.g., OIM with hPL4), the mineralized area measured in aHDFs and nHDFs was different (Figure 2), indicating that donor age influenced cellular behavior. Similar behavior was also observed with other hPLs.

Continuous passaging influences the response to osteogenic stimuli

Several studies investigated the effect of cellular senescence on osteogenic differentiation potential, but results often disagreed with each other, probably due to differing experimental conditions [20,31]. Because replicative senescence causes an increase in cell death that can affect the extent of mineral deposition, we decided to investigate the influence of different commercial hPL preparations on aging cells (i.e., cells at high passages exhibiting significantly reduced proliferation capabilities). To exclude interindividual variability, the same cell line was aged *in vitro* through serial passages, as described in Methods, and these cells were therefore compared at low and high passages.

hBM-MSCs in OIM supplemented with FBS, hPL1 and hPL2 did not differ in their deposition of minerals over time compared with cells at low passage. In the presence of hPL3 or hPL4, the amount of the mineralized area was markedly decreased at 14 days (≈4% at high passage versus ≈50% at low passage;
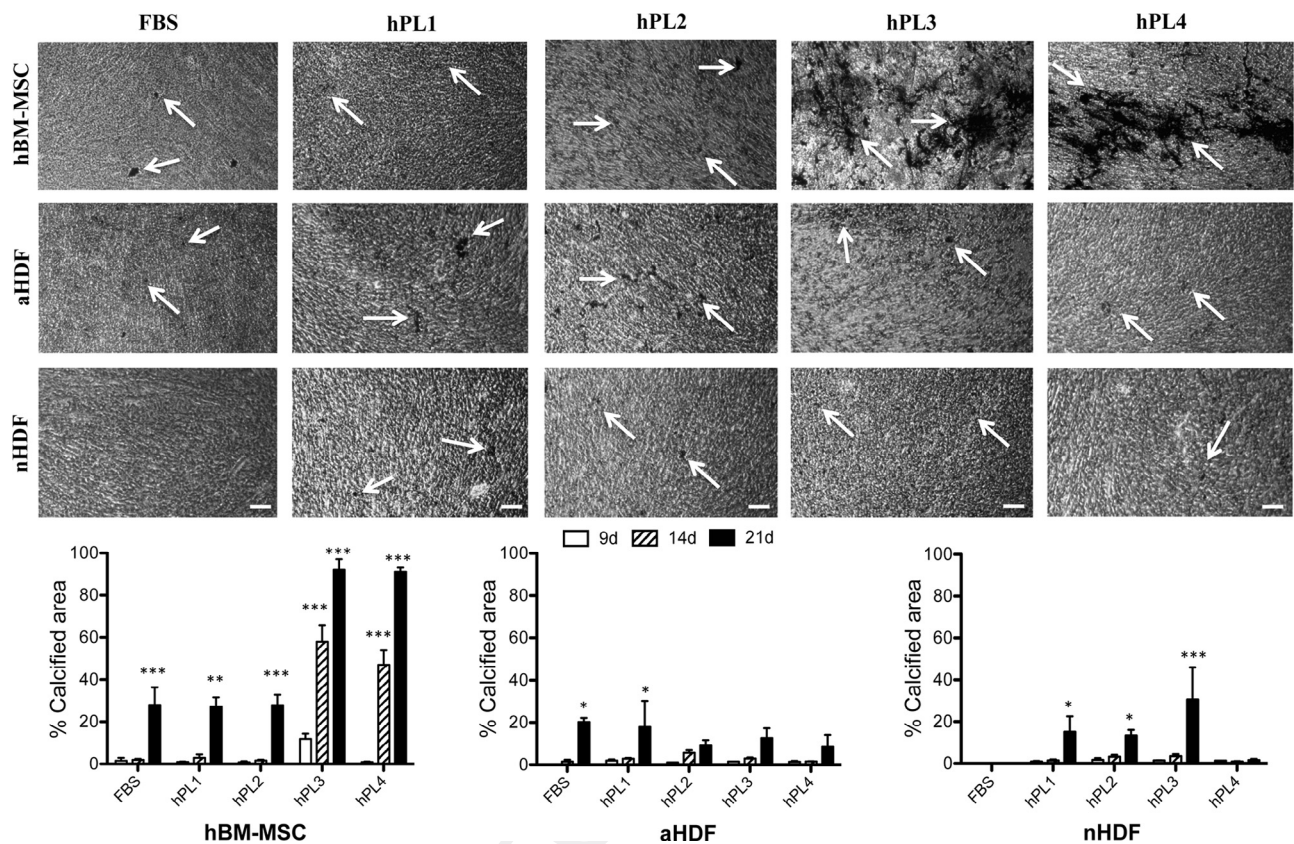
Figure 2. Calcification assay at low cell passage. Phase contrast photomicrographs of hBM-MSCs and HDFs, derived from either adult (aHDF) or neonatal (nHDF) tissues, cultured in osteogenic induction medium supplemented for 14 days with FBS or with four different commercial hPL preparations. Mineral deposits were visualized with VK staining (arrows). Bar: 120 μm. The percentage of calcified areas in hBM-MSCs, nHDFs and aHDFs cultured for 9, 14 and 21 days are shown in histograms. Results are expressed as mean values ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ 21 days versus 14 days or 14 days versus 9 days in the same cell line and with the same OIM composition.

$P < 0.001$), although calcification reached similar values at 21 days (≈84% versus ≈91% at high and low passage, respectively; Figures 2 and 3).

In aging aHDFs, VK positivity was appreciable at 14 days, especially upon hPL treatments, and at 21 days, the calcified area was >75% in all experimental conditions (Figures 2 and 3).

Continuously passaged nHDFs did not exhibit significant changes in the amount of minerals deposited over time compared with the same cell line at low passage, thus remaining poorly responsive to pro-osteogenic stimuli. The only exception was hPL3, which led to more than twice the extent of VK staining at 21 days (69% versus 31% at high and low passage, respectively; $P < 0.01$).

Discussion

The need to address the demographic burgeoning of age-related diseases and their complications has increased interest in understanding the processes governing mineralization to improve current therapeutic interventions. However, diverse *ex vivo* experimental models exploring mineralization has revealed difficulties in comparing results using various cell types and relevant reagents.

In the past decade, hPL has received significant attention for its possible clinical use and for its great effectiveness, compared with FBS, in enhancing MSC osteogenic induction. Interestingly, comparison of four hPLs showed mass spectrometry peak differences indicative of unique aspects to their composition. Identification of single components in each hPL was not within the scope of the present study, but results provide a model for future exploration to better understand which specific hPL sub-components may influence mineralization. Therefore, manufacturers' provision of pooled hPL (as we used in the present study) did not necessarily avoid diverse preparation-specific outcomes, and this likely reflected the complex nature of hPL [32]. In particular, we demonstrated that only two of the four hPLs assessed showed a significant increase of mineralized matrix compared with FBS, indicating that the extent of cell responsiveness can be markedly modulated by the physical-chemical characteristics of an hPL preparation, at least in
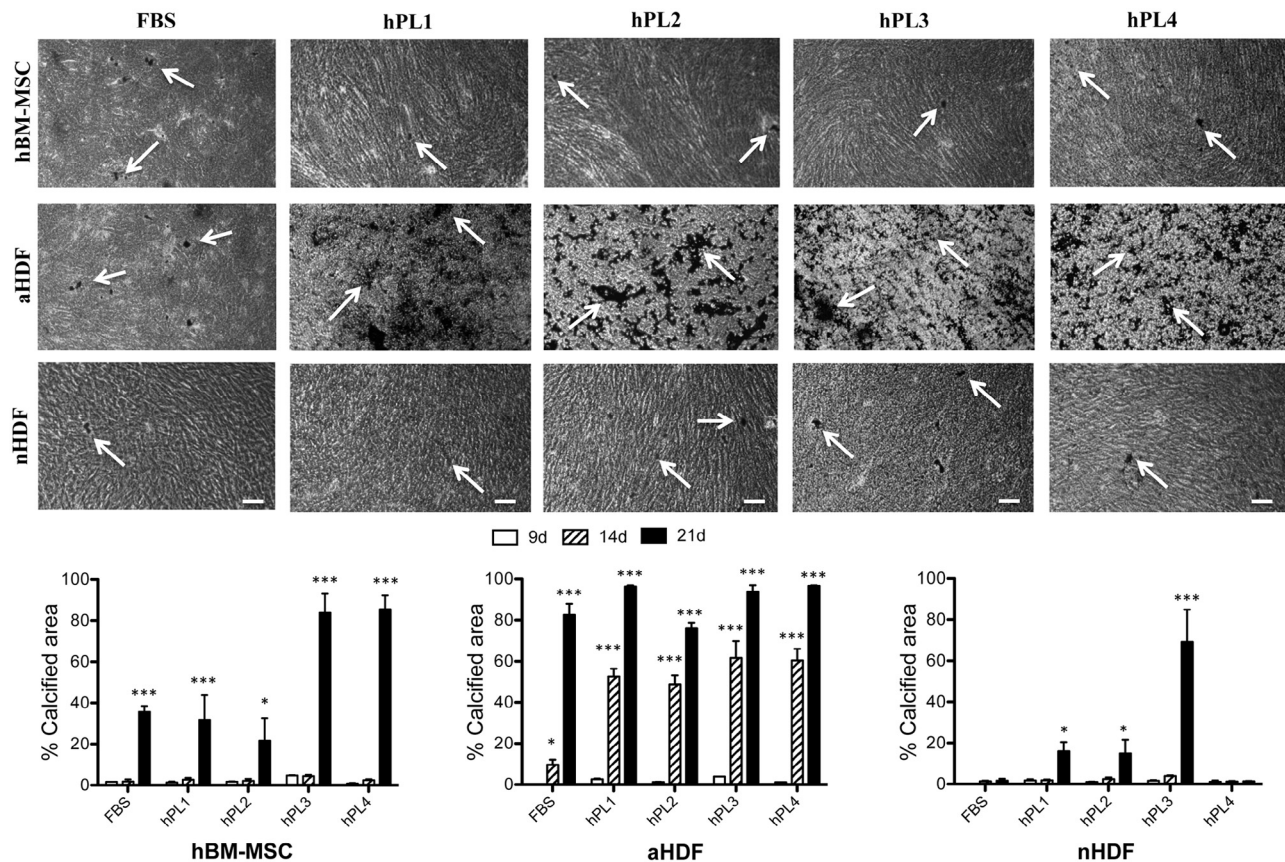
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Figure 3. Calcification assay at high cell passage. hBM-MSCs and HDFs, derived from either adult (aHDF) or neonatal (nHDF) tissues, were cultured for 14 days in OIM supplemented with FBS or four different commercial hPL preparations. Mineral deposits were visualized by phase contrast microscopy after VK staining (arrows). Bar: 120 μm. The percentage of calcified areas in hBM-MSCs, nHDFs and aHDFs cultured for 9, 14 and 21 days are shown in histograms. Results are expressed as mean values ± SEM. *$P < 0.05$; ***$P < 0.001$ 21 days versus 14 days or 14 days versus 9 days in the same cell line and with the same OIM composition.

hBM-MSCs, a mesenchymal cell type that has been extensively studied in cell culture [33] and was selected as a suitable positive control of efficient mineralization because of its high osteogenic differentiation potency. Moreover, within this context, we tested three liquid hPL forms (hPL1, hPL2 and hPL3) with differing heparin requirements versus a dry lyophilized form (hPL4) that was rehydrated before use. Heparin, a sulphated glycosaminoglycan that prevents fibrinogen conversion to fibrin as well as hPL gelatinization in the medium, is not a totally benign component. Low or high doses of heparin, for instance, can oppositely favor osteogenic differentiation [34,35]. The manufacturers of two of the hPLs (hPL1 and hPL3) recommended adding heparin to the culture medium, whereas this was not necessary for fibrinogen-depleted hPL2 or for hPL4, which already contained an anticoagulant. Our ECM calcification outcomes were not related to the requirement for heparin, as shown by near-equivalent results for hPL1 and hPL2. Interestingly, hPL4 was an adequate supplement for prompt mineralization in hBM-MSCs, as demonstrated by comparing lyophilized hPL4 with the liquid hPL formulas (hPL1, hPL2 and hPL3). Therefore, our data indicate that lyophilization of platelet extract preserves biological activity of growth factors similarly to preparations obtained after freeze–thaw cycles, sonication or activation by thrombin/CaCl$_2$ treatment [36].

With regard to the cell-specific response to pro-mineralization stimuli, dermal fibroblasts have been shown to osteogenically differentiate upon transfection of an osteogenic transcription factor [37] or when cultured in an appropriate mineralizing environment [38–41]; however, the degree of similarity between hMSCs and HDFs varies [42–44]. Of note, nHDFs and aHDFs did not mimic the prompt and dense nodular mineralization pattern of hBM-MSCs. These effects were especially evident at 14 days, with differing responses to OIM treatment, supporting the view that environmental conditions (OIM supplemented either with FBS or with one of the four hPLs) exert a different effect on the same cell type and, in addition, that the same experimental condition can induce

a different response depending on the cell type, as seen in both physiological and pathological contexts.

A peculiar feature of nHDFs was the absence of any mineralization over the time course of the experiment when using FBS, confirming previous data [19]. Substituting FBS with hPL led to nHDF-dependent ECM calcification after 21 days in three cases, whereas mineralization remained minimal when OIM was supplemented with hPL4. This does not reflect a functional inadequacy of hPL4 because the preparation supported high levels of mineralization in hBM-MSCs. In particular, hPL4 consisted of lyophilized platelet lysate from platelet-rich plasma combined with human platelet-poor plasma (1:1), and this more comprehensive supplement may have included inhibitors as well as stimulators of mineralization that combine for more cell type specific mineralization [45].

Another important point of this study is that although *in vitro* expansion is necessary to obtain a sufficient cell number for engineering and cell therapy, this requirement, having an effect on the proliferative capacity of each specific cell type, can dramatically affect the osteogenic induction efficiency. This finding was particularly evident in hBM-MSCs and in aHDFs, in which mineral deposition over time was delayed or strongly increased, respectively.

Although it is necessary to better understand the mechanisms mediating the effects of hPL on the mineralization process, this study highlights the importance of defining the production process of commercial hPLs and the release criteria, including concentration ranges of growth factors in hPL batches, so that results can be more easily compared. Additionally, it is also mandatory to take into account cellular ontology, donor age and cell proliferative capacity to optimize the osteogenic induction and design more effective cell-based therapeutic protocols.

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