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Growth and phenotypic expression of human endothelial cells cultured on a glass-reinforced hydroxyapatite

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Abstract Glass-reinforced hydroxyapatite composites (GR–HA) are bone regenerative materials that are characterized by their increased mechanical properties, when compared to synthetic hydroxyapatite. Bonelike® is a GR–HA that is a result of the addition of a $CaO–P_2O_5$ based glass to a HA matrix. This biomaterial has been successfully applied in clinical bone regenerative applications. This work aims to evaluate the ability of Bonelike® to support the adhesion, proliferation and phenotypic expression of human endothelial cells, aiming to establish new bone tissue engineering pre-endothelialization strategies. Bonelike® discs, regardless of being submitted to a pre-immersion treatment with culture medium, were seeded with first passage human umbilical vein endothelial cells, and characterized regarding proliferation and differentiation events. Pre-immersed Bonelike® allowed the adhesion, proliferation and phenotype expression of endothelial cells. Seeded materials presented positive immunofluorescent staining for PECAM-1 and a tendency for the formation of cord-like arrangements under angiogenesis-stimulating conditions, although, compared to standard culture plates, a slight decreased cell growth was observed. In this way, Bonelike® may be a suitable candidate for pre-endothelialization approaches in bone tissue engineering applications.

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1 Introduction

The current interest about the process of angiogenesis—the formation of new blood vessels from pre-existing ones—is growing in all research and clinical fields, aiming to solve several pathological conditions, including the impaired tissue regeneration. The interaction between the bone and the vascular system has long been known [1] although, for a long time, research focused on the osteogenic process. More recently, a paradigm shift was driven into the establishment of adequate vasculature given that insufficient or inappropriate tissue irrigation is associated with decreased bone formation [2]. Also, inhibition of the angiogenic process leads to the formation of fibrous tissue in animal models of bone fracture repair [3] and distraction osteogenesis [4]. It is also established that poor blood supply is a risk factor for bone healing and that several other risk factors may act negatively over the vasculature, impairing an adequate biological response [5–7].

Upon implantation of a bone graft, the first days are critical, with inflammation and revascularization occurring. Current evidence reflects that the establishment of adequate vascularisation, right after the implantation, is essential for the development of the repair process and wound healing [7, 8]. An adequate vascular network allows the nutritional support and removal of metabolic waste products from the

regenerating area, and provides a continuous availability of precursor cells to the target area, along with a large variety of biological mediators involved in cell-to-cell communication [9, 10]. In this way, endothelial cells play an essential role in the wound healing as they constitute the inner surface of the blood vessels and are the primary cells involved in the process of angiogenesis. They are also actively engaged in the release of cytokines and the expression of cell adhesion molecules, thus participating in the inflammatory response and contributing to the intercellular cross-talk. This process is particularly relevant when established within cells of the osteoblastic lineage, which are responsible for the bone formation events at the material's surface [7].

Accordingly, several approaches have emerged to enhance the rate of vascularization from the surrounding tissues into the implanted bone grafts. These include the incorporation of angiogenic factors into the materials [11, 12], deposition of an angiogenic extracellular matrix on the materials' surface [13, 14] and delivery of genes encoding angiogenic factors [15–17], all aiming to induce the endothelial function. Other promising approach is the materials' pre-vascularization with autologous endothelial cells which, upon implantation with the host's vascular system, would allow a rapid vascular supply throughout the biomaterial. Regarding this, recent in vitro studies addressed the potential of several bone regenerative materials to perform as appropriate substrates for endothelial cell growth and differentiation, to be used on tissue regeneration applications [18, 19].

New approaches on biomaterial's production for tissue engineering strategies include natural and synthetic materials, designed in a biomimetic approach regarding the bone tissue composition. Biological apatite (which comprises the mineral phase of the calcified tissues) contains mainly Ca^{2+}, PO_4^{3-} and OH^- ions, although several trace ions like Mg^{2+}, F^- and CO_3^{2-} are also present and contribute to an important biological purpose. Synthetic hydroxyapatite (HA), with the chemical composition of $Ca_{10}(PO_4)_6(OH)_2$, differs from the biological one regarding the stoichiometry, composition, crystallinity and other physical and mechanical properties [20]. Bonelike® is a glass-reinforced HA with the ability to mimic bone's inorganic chemical composition, with a microstructure composed by HA, β-tricalcium phosphate (β-TCP) and α-TCP phases spread throughout the material, creating fully interpenetrated matrices of HA and TCP [21, 22]. Bonelike® supports the proliferation and differentiation of human osteoblastic cells [23, 24] and allows fast bone formation at the bone/material interface in animal models [25, 26]. Regarding clinical application, Bonelike® grafts have been successfully applied in several areas of regenerative surgery, namely, in oral and maxillofacial surgery, implantology and orthopaedics, in a particulated form [27–30], as well as in a tridimensional macroporous scaffold, prepared by a biomodelling technique [31]. Clinical data clearly shows that Bonelike® presents appropriate features to perform as a bone graft [27–31].

Considering the relevance of angiogenic strategies in bone tissue engineering applications, the present work evaluates the ability of Bonelike® to support the adhesion, growth and differentiation of human endothelial cells. This aims to access the potential suitability of this biomaterial for pre-endothelialization approaches in order to improve graft vascularization, in bone tissue regenerative applications.

2 Experimental methods

2.1 Preparation of Bonelike®

For the production of Bonelike®, a glass was prepared with the chemical composition of $65P_2O_5$–$15CaO$–$10CaF_2$–$10Na_2O$ in mol% from reagent-grade chemicals using conventional glass making techniques. The composite was obtained by adding the milled glass to HA powder (Plasma Biotal; Tideswell UK) in 2.5% (wt/wt), using isopropanol as a solvent. The powders were mixed, dried and sieved to less than 75 μm and disc samples were prepared by uniaxial pressing at 200 MPa, using steel dies to obtain 8 mm diameter discs. The discs were then sintered at 1300°C (using a ramp rate of 4°C/min), with the temperature being maintained for 1 h, followed by natural cooling inside the furnace. Detailed description of Bonelike® preparation has been previously reported [22]. Phase identification and quantification was assessed by X-ray diffraction and Rietveld analysis.

For in vitro testing, the discs were mechanically polished to the same final topology of 1 μm using silicon carbide paper, ultrasonically degreased, cleaned with ethanol followed by deionized water and sterilized by autoclaving. Before cell seeding, Bonelike® samples were observed by scanning electron microscopy (SEM) for the assessment of the surface topography.

2.2 Culture of human endothelial cells on Bonelike®

Primary cultures of endothelial cells were established from human umbilical veins, from umbilical cords, following a standard procedure [13]. This biological material, that would be otherwise discarded, was obtained through local hospitals under the approval of the appropriate Ethical Committee, with patient informed consent.

Briefly, umbilical cords were perfused with isotonic solution to remove blood and cellular debris. Following, endothelial cells were released from the umbilical vein with

0.1% collagenase in medium M199 (7 min, 37°C, 5% CO_2/air) and the resultant cell suspension was centrifuged (1,500 rpm, 5 min). Cells were resuspended and seeded in culture plates pre-coated with 0.2% gelatine (1 h, 37°C). Cultures were established (37°C, 5% CO_2/air) in medium M199 supplemented with 20% foetal bovine serum, penicillin–streptomycin (100 UI/ml and 100 μg/ml, respectively) and 1% L-glutamine. At 70–80% confluence, primary cultures were enzymatically released (0.04% trypsin in 0.25% EDTA solution), and the obtained cell suspension was seeded (2×10^4 cells/cm^2) in standard 48-well plates (control cultures) and on the surface of Bonelike® discs, both previously coated with 0.2% gelatine. Bonelike® samples were assayed with or without being submitted to a pre-immersion treatment of 3 h, in complete culture medium. Cultures were maintained for 7 days in the medium described above, further supplemented with 1% sodium heparin and 1 μg/ml endothelial cell growth supplement (ECGS). Control and seeded Bonelike® samples were characterized throughout the culture time for cell viability/proliferation, cytoskeleton organization, expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) and ability to form tube-like networks upon the addition of a collagen type I gel.

2.3 Cell viability/proliferation: visualization of cell growth

MTT assay—reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells—was used to estimate endothelial cell viability/proliferation, during the 7 day culture period. At determined time points, samples were incubated with 0.5 mg/ml of MTT for the last 4 h of the culture period; the medium was then decanted, formazan salts were dissolved with dimethylsulphoxide and the absorbance (A) was determined at $\lambda = 600$ nm on a microplate reader. Results were expressed as A/cm^2.

Also, at days 3 and 6 of the culture period, cells were incubated with 0.1 μM calcein-acetoxymethylester (calcein-AM) for 30 min at 37°C. Calcein-AM is taken up by viable cells and converted by intracellular esterases into the membrane-impermeable fluorescent calcein that spreads throughout the entire cytoplasm of the cell. Fluorescence was visualized by confocal laser scanning microscopy (CLSM).

2.4 Immunofluorescent staining of F-actin cytoskeleton filaments and PECAM-1

At days 3 and 6, immunodetection of F-actin and PECAM-1 was conducted by CLSM. Briefly, samples were fixed with 4% formaldehyde (methanol free), permeabilized with 0.1% Triton (5 min, RT) and incubated in 10 mg/ml bovine serum albumin (BSA, 1 h, RT) with 100 μg/ml RNAse. Following, samples were either stained for F-actin or PECAM-1.

F-actin filaments were stained with Alexa-Fluor-conjugated phalloidin® (1:100, 1 h, RT) and nuclei were counterstained with 10 μg/ml propidium iodide (10 min, RT). Alternatively, samples were incubated overnight with primary PECAM-1 antibody (1:100, 4°C), followed by the addition of the secondary antibody (1:1000, anti-mouse Alexa-Fluor®; 1 h, RT), and then counterstained with 10 μg/ml propidium iodine (10 min, RT).

2.5 Culture under angiogenesis-inducing conditions

At adequate confluence (around 80% at 5 days of culture), control cultures and seeded Bonelike® discs were covered with a 1.5 mg/ml solution of collagen type I in M199 (pH ≈7.2–7.4). Within 30 min, the collagen mixture formed a gel. Complete culture medium was carefully overlayed on top of the gel and incubation was continued. Samples were analysed for cell viability/growth by calcein-AM staining, 24 h after the addition of the collagen gel.

2.6 Statistical analysis

Three independent experiments were performed using cell cultures from different patients. On the MTT assay, each point represents the mean ± standard deviation of three replicates. Statistical differences between control and Bonelike® seeded discs were analyzed by Student's *t*-test. *P*-values ≤0.05 were considered significant. Qualitative assays were performed in triplicate.

3 Results

Human endothelial cells, cultured on the surface of Bonelike® discs and on standard polystyrene culture plates, were characterized for cell viability/proliferation and differentiation events.

Bonelike® XRD data revealed a microstructure with a main crystalline phase of HA, with β- and α-TCP as secondary phases (Fig. 1a). SEM observation of the polished discs, prior to cell culture, revealed a uniform and smooth surface (Fig. 1b).

3.1 Cell viability/proliferation. Pattern of cell growth

Bonelike® samples pre-coated with gelatine, but not submitted to the pre-immersion treatment with culture medium, presented a poor performance. Cells were able to attach to the material's surface, as demonstrated by the micrograph taken at 24 h, but few cells were visible at 48 h and evident cytoskeleton modifications were visualized by

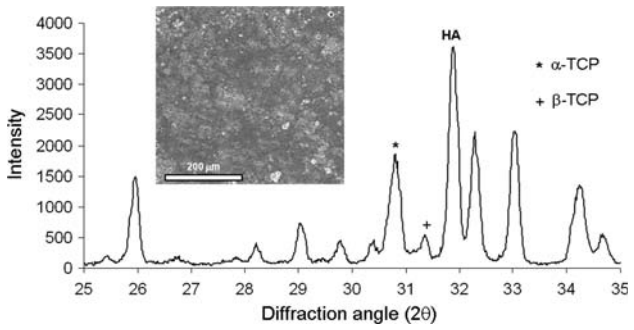

Fig. 1 X-ray diffraction pattern of Bonelike®, showing the presence of HA and α-and β-TCP phases and SEM appearance of the polished material samples (inset)

F-actin staining (Fig. 2). In addition, distributed cellular debris could be observed at 24 and 48 h of culture, on the material's surface.

CLSM observation revealed that endothelial cells attached to the pre-immersed Bonelike® samples and proliferated throughout culture time. Seeded materials, stained for F-actin cytoskeleton and nucleus, presented a tendency for a circular orientation during the proliferative phase, at 3 days of culture (Fig. 3a). Calcein-AM staining demonstrated the viability of these circular arrangements (Fig. 3b). In addition, cells presented a normal morphology, with a well defined nucleus, several cytoplasmic spreading and cell-to-cell contacts (Fig. 3c). Pattern of cell growth and cell morphology were similar to those observed on standard tissue culture plates (Fig. 3d–f).

Regarding the MTT assay (Fig. 4), cultures grown on Bonelike® samples presented an initial lag phase (during approximately 2 days) and proliferated afterwards. Compared to control cultures, MTT reduction values were lower throughout the culture time, with statistical significance at days 3 and 5.

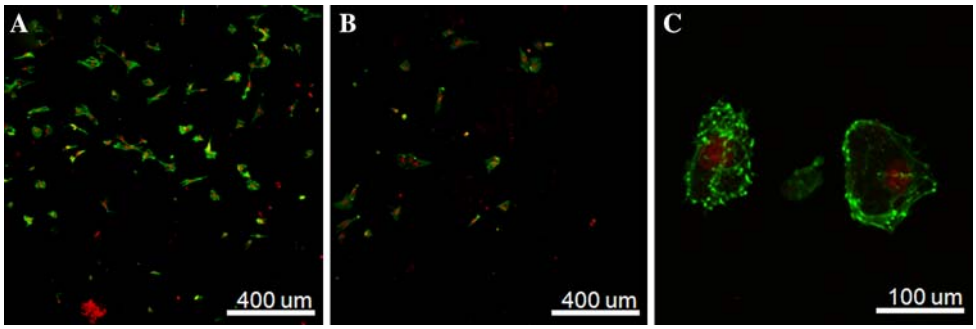

Fig. 2 Life span of human endothelial cells cultured on the surface of Bonelike® discs not submitted to the pre-immersion treatment with culture medium. Cells were able to attach to the material surface (**a**, 24 h) but died within few hours (**b**, 48 h). Also, morphological alterations were visualized by F-actin staining (**c**, 48 h)

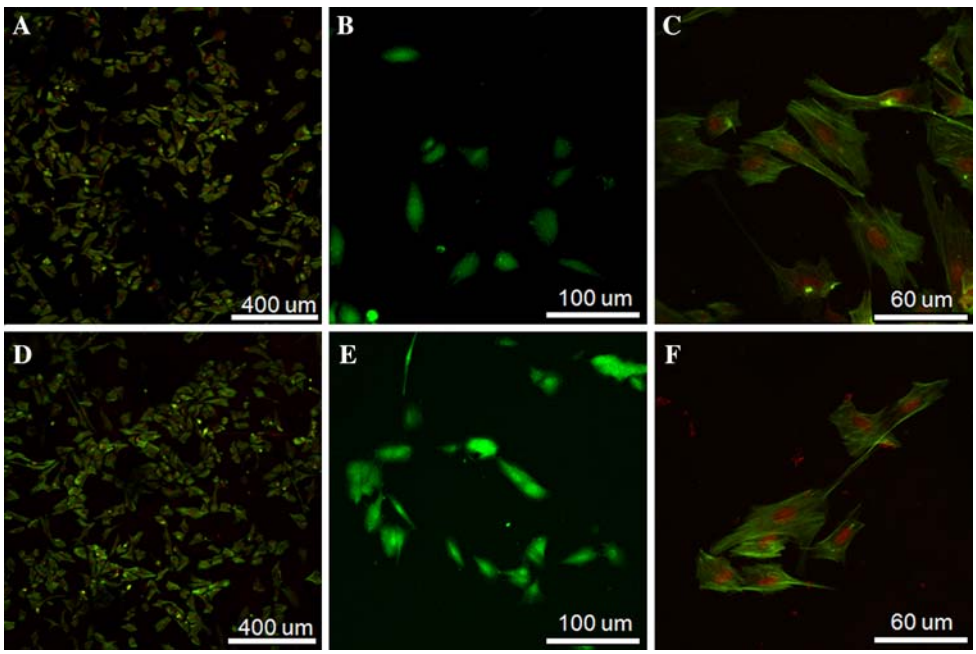

Fig. 3 Organization and morphology of human endothelial cells cultured on Bonelike® discs (**a**–**c**) and polystyrene culture plates (**d**–**f**) for 3 days. On Bonelike®, cells presented a trend for a circular organization (**a**, **b**) and a normal morphology with well-defined nucleus, cytoplasmic spreading and cell-to-cell contact. Pattern of cell organization and cell morphology were similar to those on standard culture plates (**d**–**f**). CLSM images: F-actin and nucleus staining (**a**, **c**, **d**, **f**); Calcein-AM assay (**b**, **e**)

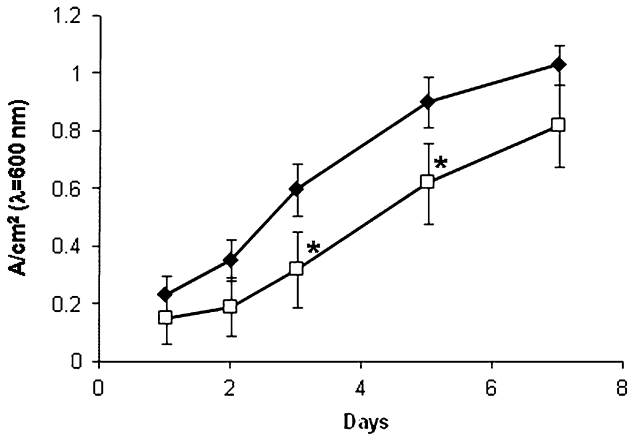

Fig. 4 Cell viability/proliferation (MTT assay) of human endothelial cells cultured on Bonelike® discs (□) and polystyrene tissue culture plates (◆)

3.2 Expression of the endothelial phenotype

Endothelial cells grown on Bonelike® samples exhibited a positive staining for PECAM-1 at the junction of adjacent cells, similar to that observed in control cultures—respectively Fig. 5a, b, d,e, regarding 6-day cultures.

Addition of a collagen type I gel to the cultures growing on Bonelike® induced a trendency for a reorganization of the cell layer with the formation of viable cord-like arrangements after 24 h, as observed in calcein-AM stained cultures (Fig. 5c). In the same experimental conditions, control cultures presented an improved organization of the collagen-induced tubular structures (Fig. 5f).

4 Discussion

Endothelial cell cultures are a valid in vitro assay to study vascular system/biomaterials interactions since they are able to mimic most steps of the complex angiogenic cascade, including cell proliferation, migration and differentiation [32, 33]. In addition, with the intimate association of the vascular system and bone being stressed, endothelial cell cultures are now considered a valuable tool in the implementation of bone tissue engineering strategies, namely regarding the possibility of biomaterials' pre-vascularization approaches [33, 34]. In the present study, Bonelike® was seeded with first passage human endothelial cells (derived from umbilical veins), that were cultured for appropriate time periods and conditions for optimal cell proliferation and differentiation [35].

Bonelike® samples assayed in the present work presented a XRD spectrum representative of this material [22]. All materials tested were coated with a gelatine solution. It has been previously reported that several biomaterials require prior coating with adhesion molecules e.g. collagen, gelatine, laminin or fibronectin, in addition to those present in the culture medium's serum component. In its absence the endothelial cell attachment is not efficient, which causes an unpredictable cellular spread and growth [36–38]. In this way, cell adhesion and proliferation were first assayed on samples pre-coated with gelatine, but not submitted to a pre-immersion treatment with culture medium (i.e. in the "as-prepared" condition). A reduced biological performance was verified with few cells attaching to the material's surface, at 48 h of culture, with

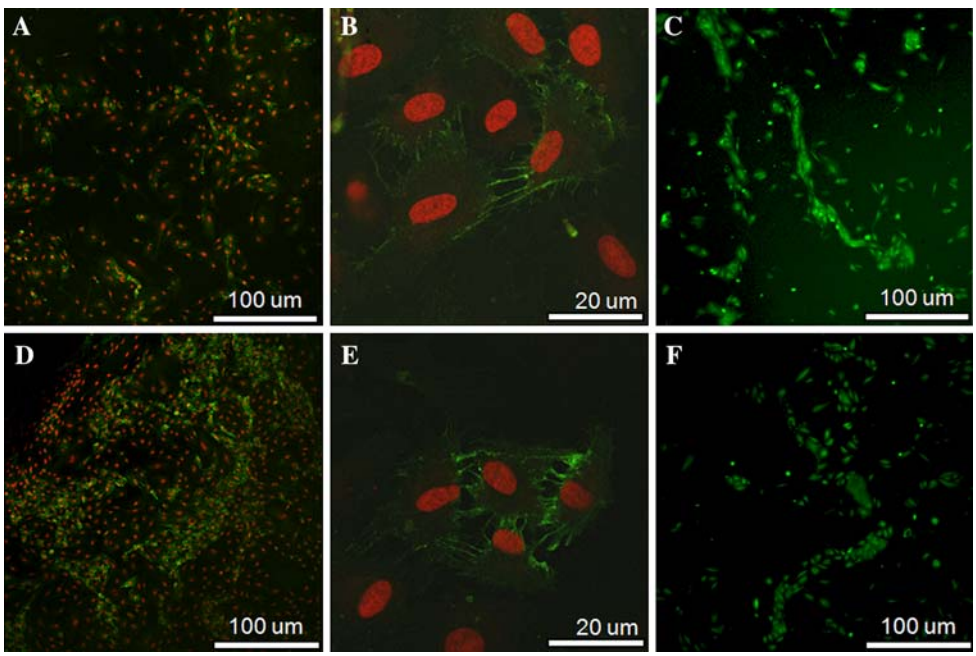

Fig. 5 Expression of functional parameters by human endothelial cells cultured on Bonelike® discs (**a**–**c**) and polystyrene culture plates (**d**–**f**), at day 6. On Bonelike®, cells exhibited a positive staining for the presence of PECAM-1 at the junction of adjacent cells (**a**, **b**) and formed cord-like arrangements upon the addition of a collagen type I gel (**c**, 24 h after the adhesion of the gel). This behaviour was similar to that observed on control cultures (**d**–**f**). CLSM images: PECAM-1 staining (**a**, **b**, **d**, **e**); Calcein-AM assay (**c**, **f**)

significant cytoskeleton modifications being visualized by F-actin staining. F-actin is highly concentrated just beneath the plasma membrane, establishing an organized layer which controls cellular shape and surface movement. It is expected to modulate cellular mechanics subjacent to the proliferation and differentiation events [39]. Early cytoskeleton modifications may compromise an adequate cell growth and phenotypic expression. Furthermore, cellular debris could be visualized by CLSM at 24 and 48 h of culture, which is in accordance with the impaired biological behaviour of non-pre-immersed samples.

Bonelike® is a glass-reinforced HA with a HA stable phase and the more soluble phases of β-TCP and α-TCP. The contact with the culture medium causes an initial superficial leaching [22] which results in a significant increase of the ionic species' concentration, on the material's surface. This impairs the anchorage of the adhering cells and has deleterious effects on the subsequent cellular events. Accordingly, improved cell behaviour was observed when Bonelike® samples were pre-immersed in the culture medium. Endothelial cells attached to the material's surface and exhibited a normal morphology with cytoplasmic spreading and multiple cell-to-cell contacts. It also showed a tendency for a circular orientation during the proliferative phase, as evident by CLSM observation of the stained samples—either with calcein-AM or F-actin. Pre-immersion of the material with culture medium caused a superficial ionic leaching and, simultaneously, deposition of bioactive molecules from the medium, apparently rendering the surface more compatible to cell attachment and growth. These dynamic events are more significant at early incubation times and tend to a progressive balance leading to a stabilization of the material's surface [22]. This probably explains the initial delay in the cell growth and the following improved cell behaviour.

Throughout the incubation time the immunofluorescent staining of PECAM-1, on seeded Bonelike® and control cultures, was performed in order to access the expression of the endothelial phenotype. PECAM-1 is a 130-kDa transmembrane glycoprotein found in large amounts on endothelial cells. It plays a major role in several cellular interactions, namely in the adhesion cascade between endothelial cells and other cell types involved in the inflammatory process, as well as, between adjacent endothelial cells during the angiogenic process [40]. These cellular junctions are crucial to maintain the endothelial layer's integrity and play an essential role in the vessel's sprouting and elongation processes [40]. According to some studies, the cellular distribution of PECAM-1 represents a very sensitive marker of the endothelial cell function [34]. Both seeded standard culture plates and Bonelike® exhibited a positive localized staining at the junction of adjacent endothelial cells. Under the established culture conditions, the cells were spread flatly and the PECAM-1 concentration along the borders highlights the established cell–cell contacts. In addition, phenotypic characterization was conducted at angiogenesis-stimulating conditions, i.e. upon the addition of a collagen type I gel. Cells growing on standard tissue culture plates showed an evident tendency for reorganization, with the formation of cord-like structures, in agreement with it has been reported regarding the in vitro behaviour of endothelial cells [41, 42]. Seeded Bonelike® presented a similar tendency, an effect independent of the subjacent surface topography considering the SEM appearance of the material's surface, before cell seeding. In both situations these structures maintained their viability, as shown by calcein-AM assay. However, Bonelike® showed a poorer definition of these structures, which might be related with the surface reactive properties, previously mentioned.

Results showed that the in vitro performance of Bonelike®, regarding endothelial cell culture, was similar to the one occurring in standard tissue culture plates, although with an initial impairment regarding cell proliferation. Accordingly and aiming to establish new vascularization strategies, the behaviour of human endothelial cells grown over bone regenerative biomaterials was addressed in few recent studies. Choong et al. [18] showed that hydroxyapatite-coated polycaprolactone substrates were superior for human bone marrow endothelial cell line (HBMEC-60) attachment and proliferation, when compared to untreated substrates. Unger et al. [38] reported that human dermal microvascular endothelial cells (HDMEC), growing in angiogenesis' stimulating conditions on three-dimensional porous polyethersulfone (PES) fibres, formed microvessel-like structures similar to those attained on tissue culture plates and in vivo. In the present work, rudimental cord-like arrangements could be observed on Bonelike® samples. Established differences with the reported studies might account for these observations, namely those related to the in vitro system, i.e. endothelial cell type, cell passage, cell plating density and, also, differences regarding the biomaterials tested and pre-treatments carried out. Endothelial cells are very sensitive to the substrates' surface properties, chemistry, structure and porosity. These characteristics are known to affect cell adhesion, spreading, growth and function [43–45].

The present results provide information regarding the ability of Bonelike® to support endothelial cells' adhesion, proliferation and differentiation. However, due to the known complex interactions between endothelial and osteoblastic cells in the regenerative environment [7, 33, 34], as clearly evidenced in co-culture systems [34, 35], the suitability of Bonelike® for endothelialization strategies needs to be addressed in more complex and representative models.

5 Conclusion

Bonelike® allowed the adhesion and spreading of human endothelial cells. Also, this bone graft biomaterial supported the subsequent proliferation and phenotypic expression, with growing cells showing positive immunofluorescent staining for PECAM-1 and a trend for the formation of cord-like arrangements under angiogenesis-stimulating conditions. The surface reactive properties of Bonelike® are compatible with the proliferation and differentiation of endothelial cells, a relevant cell type involved in the bone regenerative process, suggesting the potential suitability of this biomaterial for pre-endothelialization strategies in bone tissue engineering.

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