Analysing the cell response to biomaterials for spinal fusion surgery

Minor Research Project
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PART 1 – Osteostimulation of BCP<µm-Collagen

Abstract

More than 2,2 million bone grafting procedures are accomplished every year to treat bone defects and perform spinal fusion surgeries. To date, the gold standard treatment to achieve bone regeneration and new-bone deposition is the Autologous Bone Graft (ABG), a technique that suffers from limited availability and the need for an additional surgical operation. To overcome these limitations, strategies to augment the overall osteoinductivity of synthetic bone grafts have been developed. Among them, strategies aiming to promote bone regeneration through the immune system have reached the forefront of bone regeneration. In fact, immune cells can promote the local release of soluble factors that recruit bone precursor cells and promote their differentiation towards osteoblasts. Recently, a new biphasic calcium phosphate with sub-micron needle-shaped surface topography (BCP<µm) has been developed at Kuros Biosciences capable of promoting macrophage polarization to the M2 phenotype, which has been associated with augmented bone regeneration. BCP<µm granules has shown comparable effects to ABG in vivo; however, it is difficult to implant due to its granule morphology. Hence, a new formulation has been developed consisting of BCP<µm granules embedded in a hydratable collagen matrix (BCP<µm-Collagen). Since the exposure of the needles is key to BCP<µm granules bioactivity, in this study we investigated whether the collage matrix hinders granules' function. To do so, we cultured hMSCs on the surface of BCP<µm-Collagen to assess their metabolic activity and osteogenic differentiation, and to compare their response to a predicate device consisting of discs of BCP<µm. The Alamar blue assay on hMSCs cultured on the surface of the BCP<µm discs or BCP<µm-Collagen for 24 hours showed up to 20-fold increase in metabolic activity. Moreover, ALP and DNA quantification after 10 days of stimulation with OM showed up to 2-fold increase in ALP production per cell. No differences in DNA content of hMSCs were observed between BCP<µm discs or BCP<µm-Collagen after 10 days of culture. These results suggest that collagen substantially favours cell attachment and metabolic activity within the first 24 hours of hMSC culture. Moreover, an increase in osteogenic differentiation towards osteoblast was reported in BCP<µm-Collagen. Hence, the addition of Collagen to BCP<µm granules does not hinder Granules' function and BCP<µm-Collagen represents a promising bone filler with osteostimulating properties.

Definitions and Abbreviations

hMSC	Human Mesenchymal Stem cells
OM	Osteogenic Media
BM	Basal Media
ALP	Alkaline Phosphatase
SEM	Scanning Electron Microscopy
BCP<µm	Biphasic calcium phosphate granules with submicron surface topography
BCP<µm-Collagen	BCP<µm granules embedded in a collagen matrix
BCP<µm disc	BCP<µm granules fabricated into disc shape

Introduction

Following excessive bone loss and during spine fusion surgeries, a significant amount of new bone is needed to fill the damaged tissue or to fix the spine. Currently, the gold standard treatment

to treat bone voids is the Autologous Bone Graft (ABG) [1]. More than 2,2 million bone grafting surgeries are performed every year, and the efficacy of the ABG treatment resides in its osteoconductive, osteoinductive, histocompatible, and non-immunogenic properties [2]. However, ABG suffers from limitations including limited availability and the need for an additional surgical operation [3]. For these reasons, new alternative treatments are being developed, such as the implantation of synthetic grafts harnessing regenerative properties [4–6].

Mesenchymal stem cells (MSCs) are the precursors of osteoblasts, the effectors of bone deposition, and they have been investigated in several cell-based treatments for bone regeneration thanks to their high proliferation, multilineage potential, anti-inflammatory, and immune regulatory properties [7]. However, strategies solely relying on augmenting hMSC osteogenic differentiation usually fail once they reach the clinics [8]. This highlights the need for a better understanding of bone homeostasis and regeneration to develop more reliable treatments.

Recent studies demonstrated that osteoimmunomodulatory bone fillers with submicron topography showed effective bioactivity and bone healing properties, mainly due to the mechanoresponse of macrophages to the surface topography of the material leading to their polarization towards a regenerative phenotype (M2) [9–11]. In fact, M2 macrophages release cytokine and soluble factors resulting in the recruitment and osteogenic differentiation of hMSCs [12]. Although the exact mechanisms and factors underlying these observations are still unknown, these observations have brought osteoimmunology to the forefront of bone regeneration [4,11,13–15].

Despite the main role of the immune system in assisting bone regeneration, hMSCs cultured on synthetic bone fillers showed increased attachment, proliferation, and osteogenic differentiation; hence, the response of hMSCs to the biomaterial also contributes to the outcome of the treatment [7,16,17]. For these reasons, investigating the interactions lying in the interface between bone grafts, hMSCs, and immune cells is of critical importance to better predict the *in vivo* outcome of the materials.

BCP<µm granules are an osoteimmunomodulatory biphasic calcium phosphate (BCP) with submicron needle-shaped surface topography developed for spinal fusion surgery. In vitro, in vivo, and clinical investigations of BCP<µm showed efficient bone formation when implanted orthotopically or ectopically as an ABG extender, and macrophages cultured on the surface of BCP<µm showed polarization towards the pro-healing M2 phenotype [9,11,18]. Moreover, hMSCs cultured on the surface of BCP<µm granules showed osteogenic differentiation during internal investigations at Kuros Biosciences in P17021. Although very effective in promoting bone formation, BCP<µm granules are difficult to implant for the surgeon, hence a new generation of BCP<µm granules has been developed, named BCP<µm-Collagen. It consists of BCP<µm granules embedded in a collagen matrix to allow hydration with bone marrow aspirate or blood, improved moldability, and prevent the diffusion of the Granules in unwanted sites. Since the needle-shaped surface topography of the Granules is key for their bioactive functions, in this study we will evaluate whether the presence of a collagen binder hinders Granule's functions in promoting hMSC attachment, and osteogenic differentiation. To do so, we will compare the response of hMSCs to BCP<µm-Collagen with the predicate device BCP<µm granules in terms of attachment, proliferation, metabolic activity, and osteogenic differentiation.

Materials and Methods

Preparation of BCP<µm discs

Calcium phosphate powder (Kuros Biosciences B.V.) was sintered into discs to allow for easier cell culture (Figure 1). As the process is protected by a patent, it can only be described generally. In brief, calcium phosphate powder was weighted, mixed with water and ethanol, and stirred to obtain a slurry. The slurry was then sintered at high temperatures to obtain blocks of calcium phosphate. The blocks were then cut into a rectangular parallelepiped and smoothed into cylinders of 14 mm diameter. The cylinders were finally cut into discs of 2 mm thickness through a rotating blade and autoclaved to obtain the needle-shaped surface topography characteristic of BCP<µm . The discs were

then stored in sterile containers until use. BCP<µm discs were pre-incubated in 2 mL of Basal Media (BM) consisting of aMEM GlutaMax supplement no ribonucleoside (Gibco™ – 12549089) supplemented with 10% (v/v) heat-inactivated FBS (Gibco™ - A4766801), 100 units/mL 100ug/mL PenStrep (ThermoFisher™ - 15140148), 0.2 mM Ascorbic Acid 2-Phosphate (Sigma-Aldrich™ - A8960) for at least 4 hours before starting the experiment.

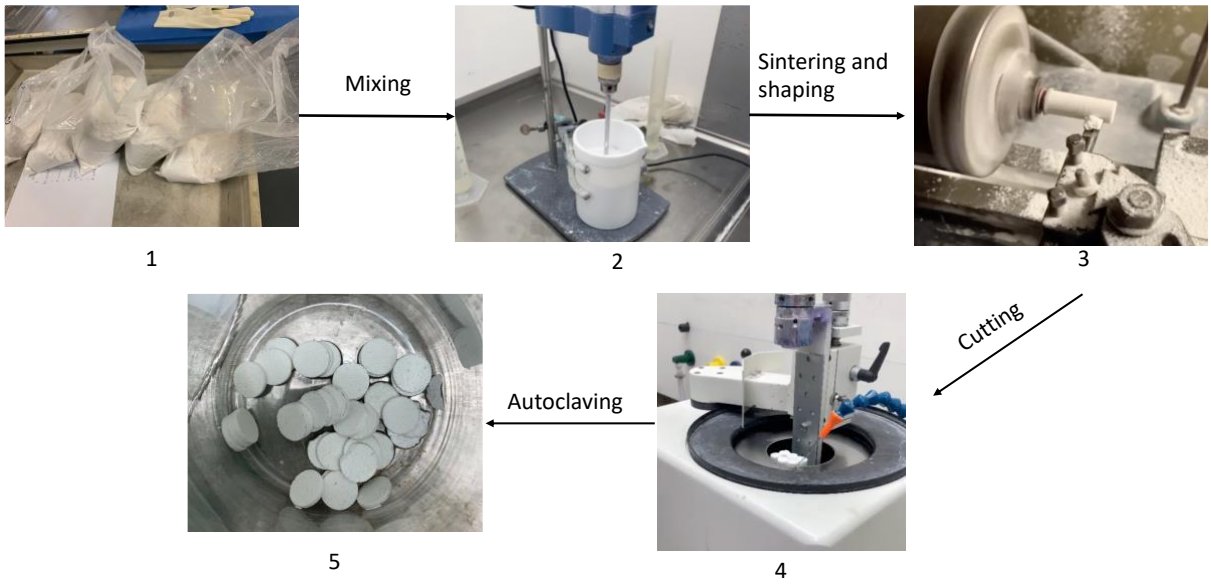

Figure 1. Schematic representation of the preparation of BCP<µm discs for cell culture. To generate BCP<µm discs, calcium phosphate powders were mixed (1), turned into a slurry (2), sintered to obtain blocks of calcium phosphate, and shaped into cylinders of 14 mm diameter (3). After that, the cylinders were cut into discs of 2mm thickness (4) and autoclaved to obtain the needle-shaped surface topography (5).

Cell culture and seeding on the materials

Three donors of hMSCs between passage 3 and 4 (D1: 17-021-BM, D2: 17-018-BM, and D3: 15-048-BM) were thawed and expanded in T175 flasks until 70% confluency in BM. Once 70% confluency was reached, hMSCs were detached with trypsin-EDTA (ThermoFisher™ – 25300054), centrifuged at 300 g, and counted. Then, the media was then removed from the materials, the cell suspension was adjusted to $1x10^6$ cells/mL and 150µl of solution was seeded on the surface of the materials (~1,5 x 10^5 cells/material). After 2h incubation at 37°C in a humidified atmosphere containing 5% CO_2 to allow cell attachment, 2 mL of Osteogenic Media (OM) consisting of BM supplemented with 10 nM Dexamethasone (Sigma-Aldrich™ - D8893) was added to the cells. Half of the media was replaced every 2-3 days for 10 days.

Evaluation of BCP<µm discs for cell culture

Two discs per group were collected after 24 hours and 10 days of culture with hMSCs to assess cell attachment, morphological changes, and proliferation through methylene blue staining and SEM imaging (Figure 2).

For proliferation, samples were fixed with 4% PFA, stained with Methylene blue, and imaged with an optical microscope. Quantification of the area covered by the cells at 24 hours and 10 days was performed using CellProfiler software for image analysis. In brief, the total area of the discs was identified by masking the pores and removing them from the image, then the dark blue areas of the discs corresponding to the cells were identified by thresholding, and they were converted into objects. The area occupied by the cells was finally calculated as a percentage of the total area.

For attachment and morphological evaluation, the samples were fixed in 4% PFA, dehydrated in 60%, 70%, 80% 90%, 95%, and 100% ethanol for at least 2 hrs for each step. Then, the samples were transferred to a 1:2 solution of HMDS:100% ethanol for 20 minutes, and a 2:1 solution of HMDS:100% ethanol for 20 minutes. After that, the samples were transferred into 100% HMDS for 20 minutes twice, and the HMDS was then left to evaporate overnight in a fume hood. Finally, the samples were gold-sputtered and observed with SEM at 350x and 600x magnification.

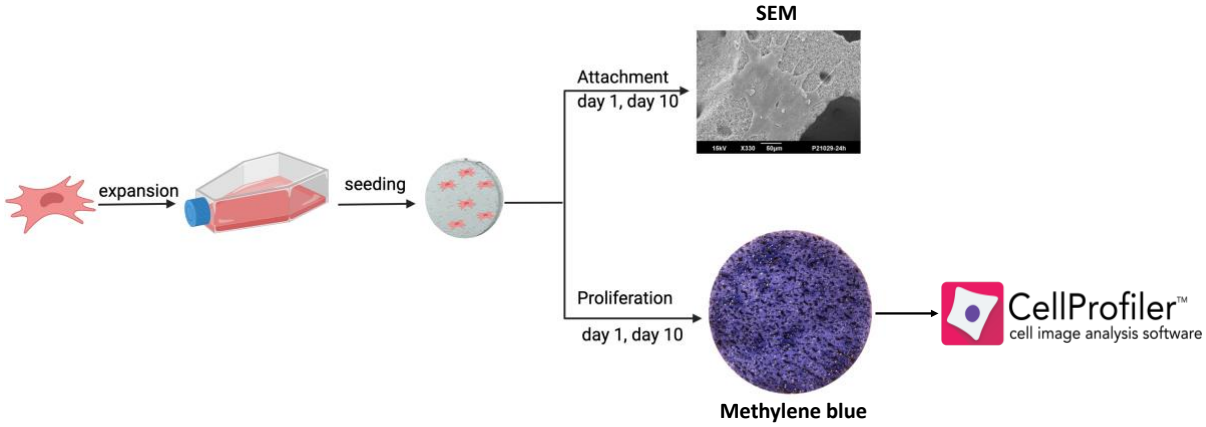

Figure 2. Overview of the experiments to assess the suitability of BCP<µm discs for cell culture. hMSCs were expanded and seeded on the surface of BCP<µm discs. After 24 hours and 10 days of culture in OM, hMSCs were fixed, and underwent SEM analysis and Methylene blue staining to evaluate hMSC attachment and proliferation to the surface of the BCP<µm discs respectively. The quantification of the progressive coverage of the surface of the BCP<µm discs by the hMSCs over time was performed with CellProfiler.

BCP<µm-Collagen and BCP<µm discs preparation for Osteostimulation study

BCP<µm-Collagen (Kuros B.V.) devices were used as received and cut into pieces of approximately 0.2g - 0.25g to obtain a similar volume to BCP<µm discs. Two devices of BCP<µm discs or BCP<µm-Collagen were then pre-incubated in BM for at least 4 hours at 37°C in a humidified atmosphere containing 5% CO_2.

Osteostimulation study of BCP<µm-Collagen and BCP<µm discs

Part of the hMSC suspension was adjusted to $1x10^6$ cells/mL and 150µl of solution was seeded on the surface of the materials (~$1,5x10^3$ cells/material) in BM. The remaining cells were adjusted to $2.5x10^4$ cells/mL in BM and 200 µL were seeded in a 96-well plate ($1,5x10^4$ cells/cm^2). After 2h incubation at 37°C in a humidified atmosphere containing 5% CO_2 to allow cell attachment on the materials, 2 mL of OM were added to the materials. Once hMSCs seeded on 96-well plates reached 70% confluency, the media was aspirated and 200 µL of either BM or OM were added to the wells as negative and positive controls, respectively. Half of the media was replaced every 2-3 days for 10 days on both plates and materials. Two materials per group were collected after 10 days to evaluate ALP and DNA content. To do so, hMSCs on both materials and controls were lysed using 0.1% Triton X-100/PBS under agitation for 30 min at room temperature, and the lysate was stored at -80°C until analysed. The ALP content was quantified using a colorimetric ALP assay kit (Abcam™ - ab83369) and Em. 430 nm following manufacturer's instructions. The DNA content was quantified using CyQuant dsDNA kit (Promega™ - E2670) in fluorescence at Ex. 483 nm and Em. 530 nm following manufacturer's instructions. The ALP content was then normalised for DNA. An overview of the experiment is shown in Figure 3.

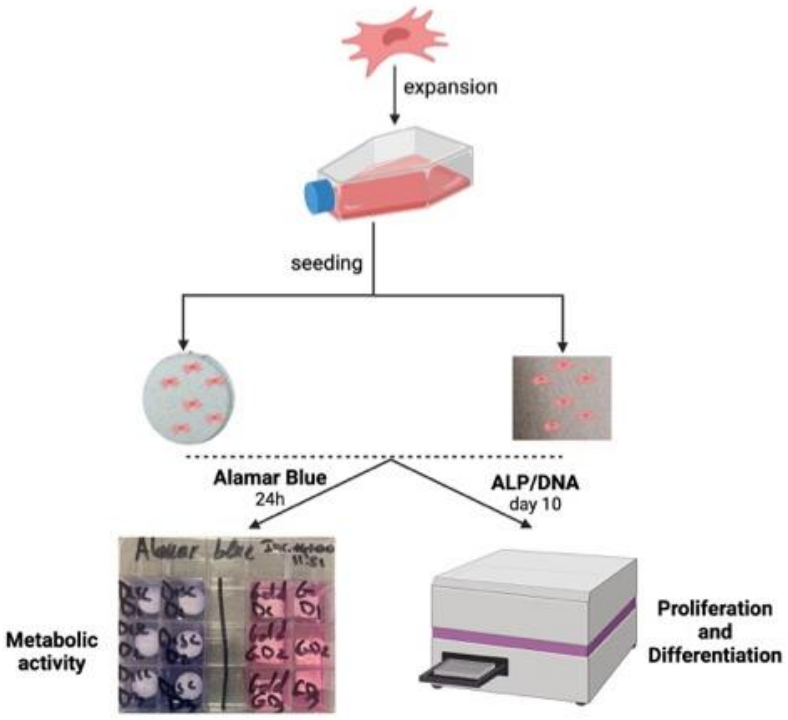

Figure 3. Overview of the experiments to assess the suitability of BCP<µm discs for cell culture. hMSCs were expanded and seeded on the surface of BCP<µm discs or BCP<µm-Collagen. After 24 hours of culture, Alamar blue assay was used to assess hMSC metabolic activity. After 10 days of culture, the materials were harvested to quantify DNA and ALP content.

Alamar blue assay on BCP<µm discs and BCP<µm-Collagen

After 24 hours incubation of hMSCs seeded on the surface of either BCP<µm discs or BCP<µm-Collagen, the media was aspirated, the Alamar blue (ThermoFisher™ – 10161053) was diluted 10 times in OM, and 1 mL of Alamar blue solution was added to the wells with either BCP<µm discs or BCP<µm-Collagen. The materials were then incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2 protected from light. The media was finally collected in duplicates and stored at 4°C protected from light until analysed using a microplate reader with Ex. 544 nm and Em. 620 nm filters. An overview of the experiment is shown in Figure 3.

Results

Evaluation of BCP<µm discs for cell culture

Two discs per group were collected after 24 hours and 10 days of culture with hMSCs in OM on the surface of BCP<µm discs to assess cell attachment and proliferation through methylene blue staining and SEM imaging. For proliferation, samples were fixed, stained with methylene blue, and imaged with an optical microscope (Figure 4).

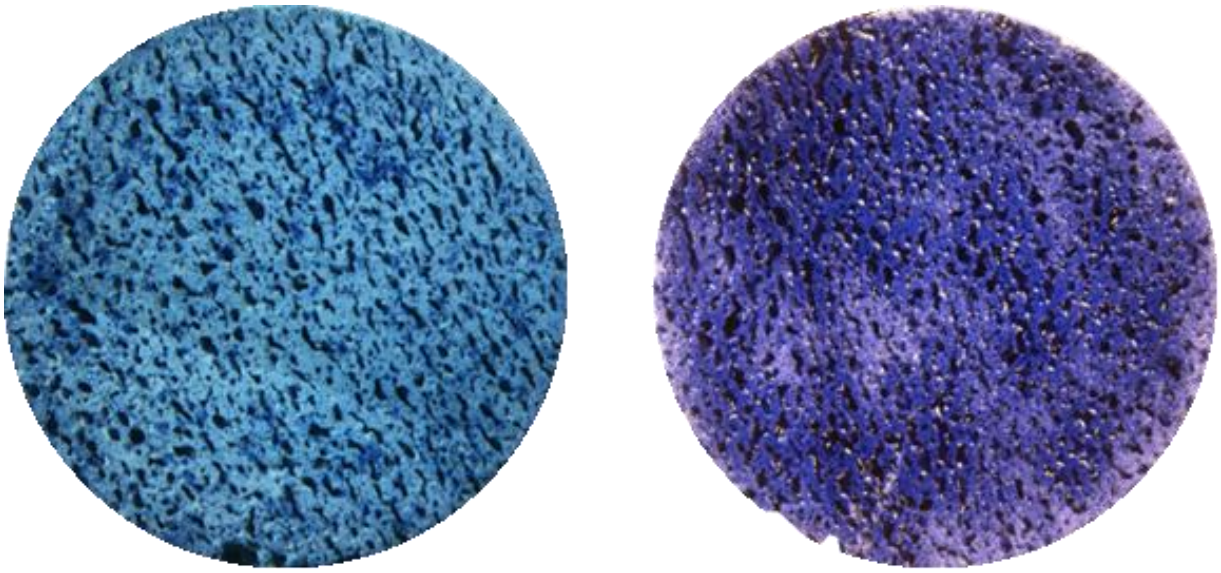

Figure 4. Representative images of hMSCs fixed on the surface of BCP<µm discs. BCP<µm discs were collected 24 hours (left) or 10 days (right) after seeding, hMSCs were fixed on the surface of BCP<µm discs and stained with Methylene blue. The regions that are stained dark blue represent the cells.

Quantification of the area covered by the cells at 24 hours and 10 days was performed using CellProfiler software for image analysis. An overview of the pipeline developed for cell identification and quantification is shown in Figure 5.

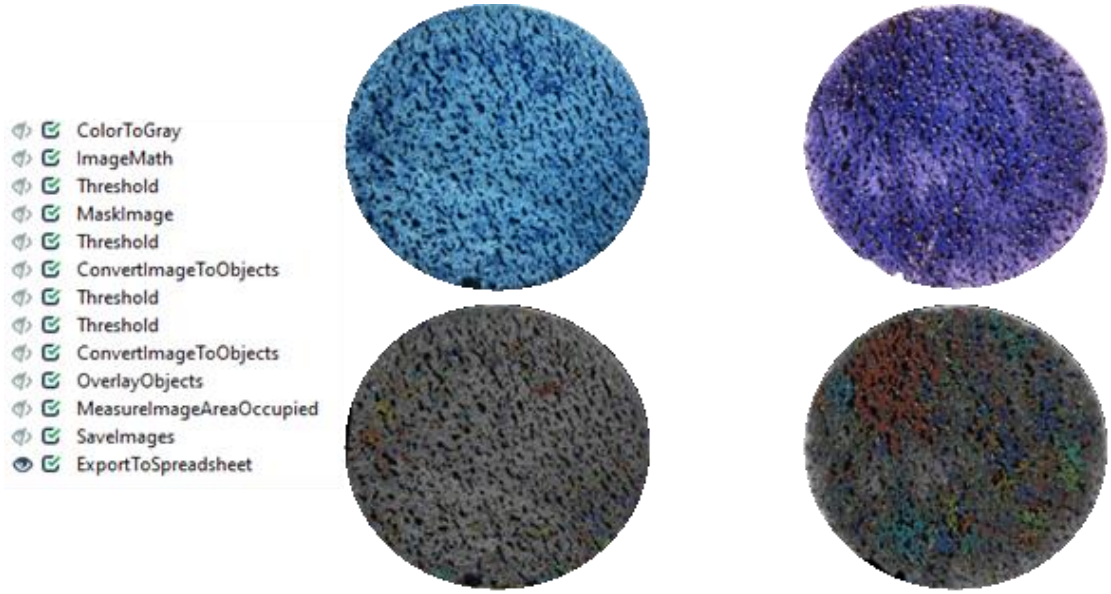

Figure 5. Representation of the pipeline used for the quantification of the area occupied by hMSCs at 24 hours (left) and 10 days (right) after seeding. The discs on top represent the images acquired with the optical microscope, and on the bottom are the discs after the analysis with CellProfiler. The colours correspond to the cells identified by CellProfiler; different colours represent non-adjacent hMSCs.

The analysis revealed an equal number of cells between the donors at 24 hours suggesting a satisfactory performance of the pipeline in identifying the cells. A 2- to 3-fold increase in the coverage

of the discs' surface by the hMSCs in 2 out of 3 donors was observed after 10 days of culture in OM (Figure 6). Hence, hMSCs seeded on the surface of BCP<µm discs for 10 days progressively cover up to 50% of the surface of the disc.

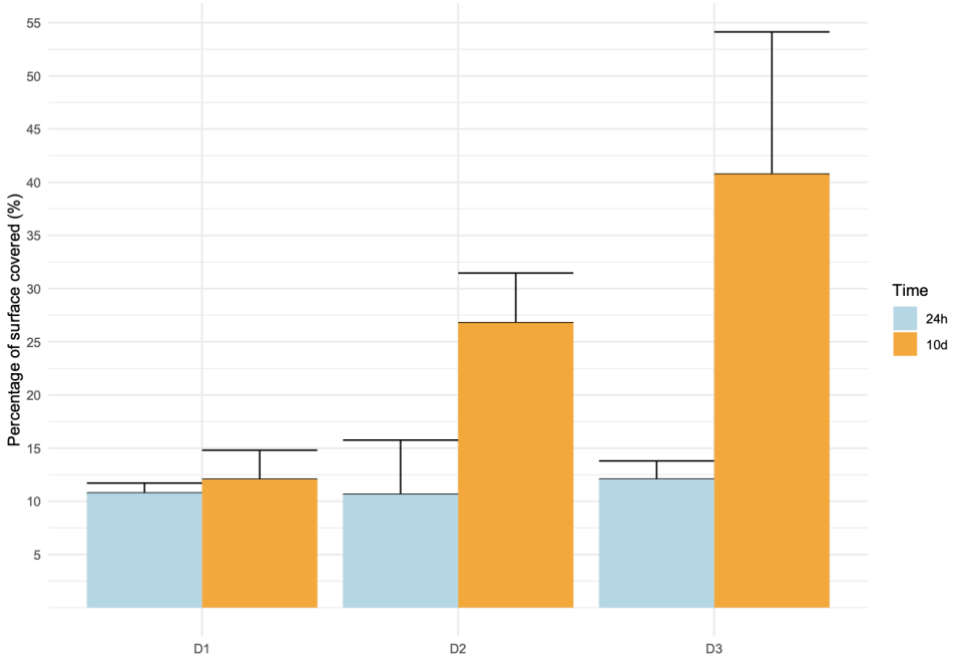

Figure 6. Quantification of the area covered by hMSCs seeded on the surface of BCP<µm discs over time. The percentage of the total area of the surface of the discs occupied by three donors of hMSCs after 24 hours (light blue) or 10 days (orange) after seeding in OM. Data are shown as mean ± standard deviation.

For attachment evaluation, the discs were dehydrated, coated in gold, and imaged with SEM. The analysis revealed progressive coverage of the disc by the cells from 24 hours to 10 days, and morphological changes of the hMSCs in time. In particular, the cells shifted from a flat morphology to an elongated one typical of cell adhesion and osteogenic differentiation (Figure 7).

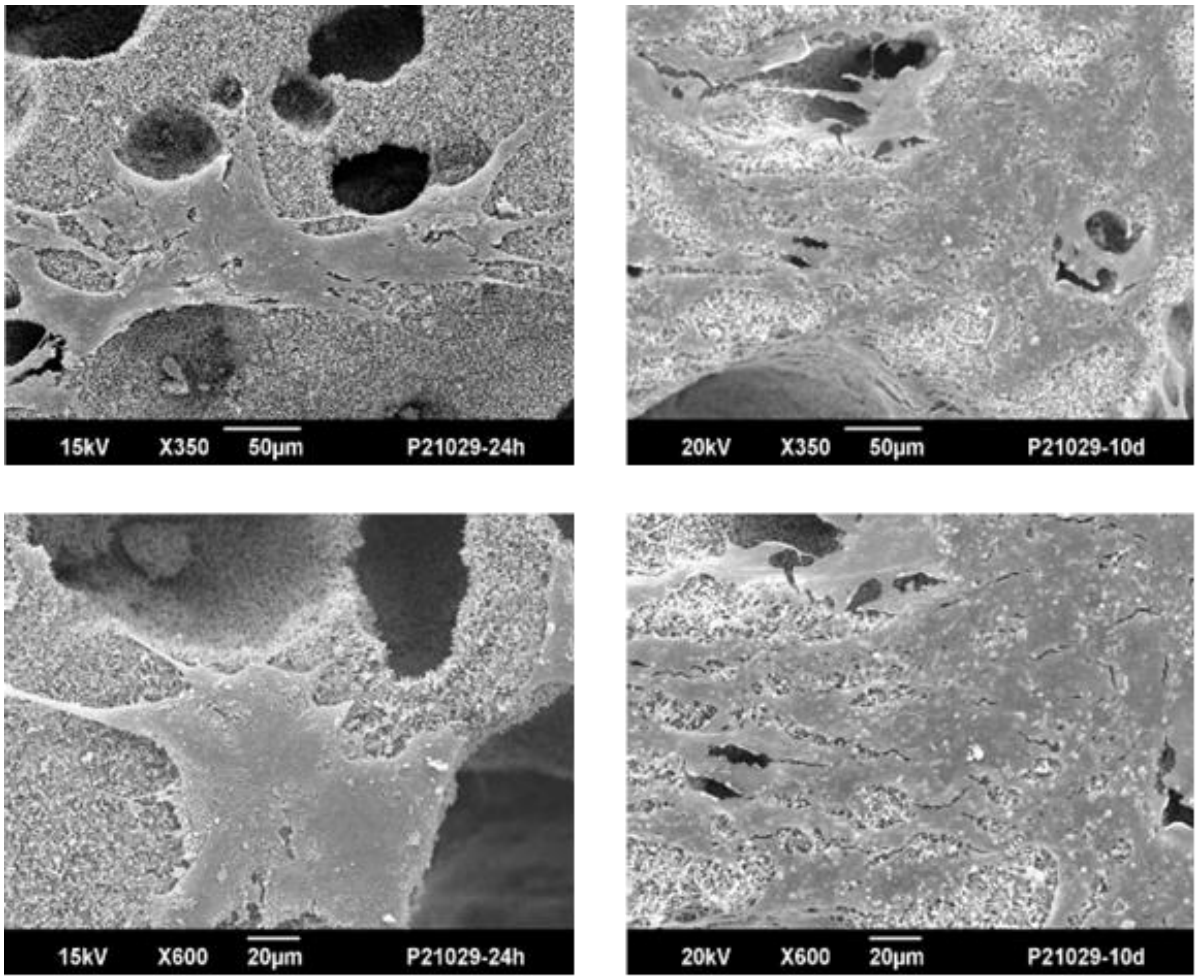

Figure 7. Representative images of hMSCs attached to the surface of BCP<µm discs. BCP<µm discs were collected after 24 hours (left) or 10 days (right) of culture with hMSCs in OM, dehydrated, gold-sputtered, and imaged. Images are acquired at low (top) and high (bottom) magnification.

These results suggest that hMSCs cultured on the surface of the BCP<µm discs proliferate over time and shift from a flat morphology towards an elongated one. In particular, hMSCs cover around 10% of the area of the disc after 24 hours and up to 30-50% of the area after 10 days of culture in OM. However, hMSC proliferation was only observed in 2 out of 3 of the donors tested. Moreover, the evaluation of the attachment of the hMSCs to the surface of the discs revealed that the cells appear isolated and flat after 24 hours, whereas they form a continuous layer with elongated morphology after 10 days. This confirms the proliferation of the cells on the surface of the material and indicates attachment and potential osteogenic differentiation towards osteoblasts. Driven by these results, the discs were considered suitable for cell culture and were further employed to compare their osteogenic potential with BCP<µm-Collagen.

Alamar blue assay on BCP<µm discs and BCP<µm-Collagen

After 24 hours of hMSC culture on the surface of BCP<µm discs or BCP<µm-Collagen, Alamar blue media was diluted 10 times in OM and 1 mL was added to the materials for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. The media was then collected in duplicates and analysed using a microplate reader with Ex. 544 nm and Em. 620 nm filters. The quantification revealed up to a 20-fold increase in metabolic activity of hMSCs when cultured on the surface of BCP<µm-Collagen compared to BCP<µm discs for 24 hours (Figure 8).

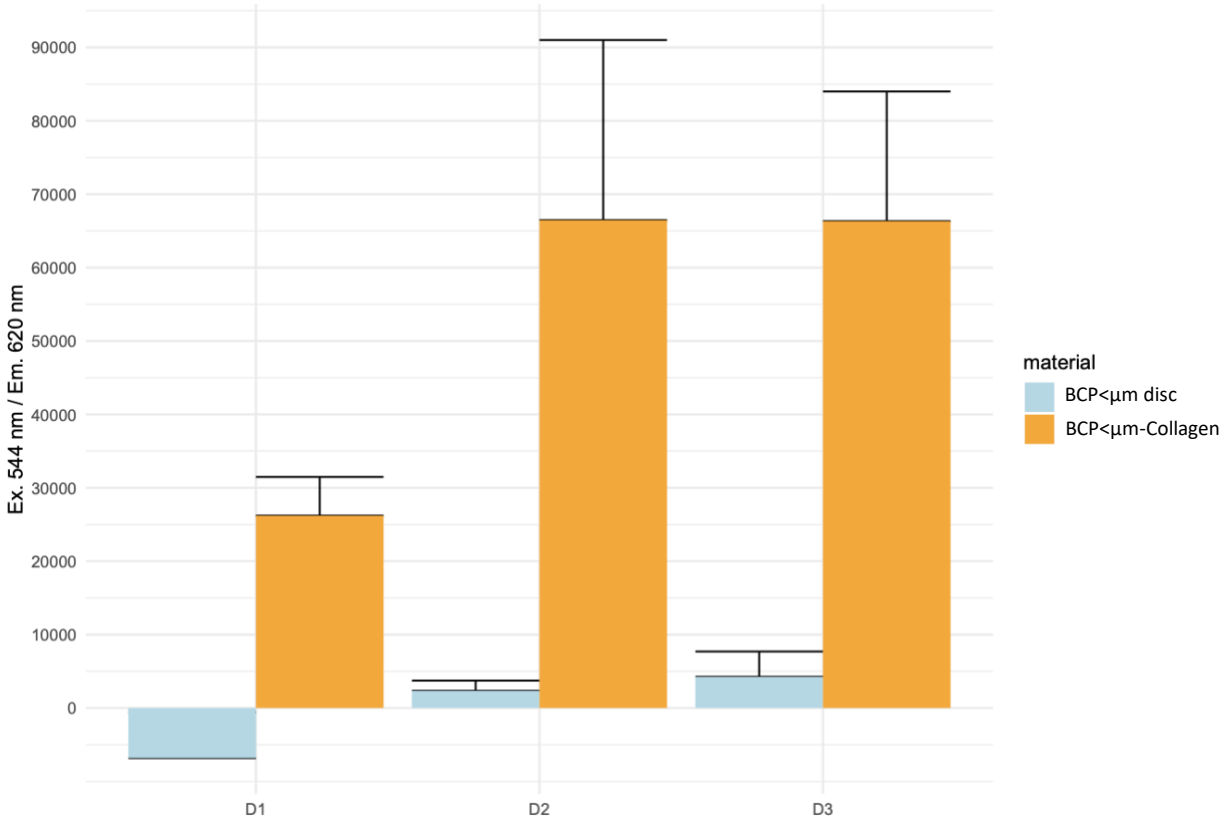

Figure 8. Alamar blue assay on BCP<µm discs or BCP<µm-Collagen after 24 hours of culture with hMSCs. After 24 hours of culture on the surface of BCP<µm discs (light blue) or BCP<µm-Collagen (orange), Alamar blue was added to the cells for 4 hours and the reduced Alamar blue was quantified in fluorescence using Ex. 544 nm and Em. 620 nm filers. Data are shown as mean ± standard deviation.

Osteostimulation study on BCP<µm discs and BCP<µm-Collagen

Three donors of hMSCs were seeded on the surface of BCP<µm discs or BCP<µm-Collagen and cultured for 10 days. The quantification of DNA after 10 days of culture in OM revealed a comparable amount of DNA between BCP<µm discs and BCP<µm-Collagen in two out of three donors (Figure 9). For one donor, a 4-fold increase in DNA count was observed when cultured on the surface of BCP<µm-Collagen. As expected, an overall increase in DNA content was observed when hMSCs were cultured on the materials compared to the 96-well plates (23-fold increase).

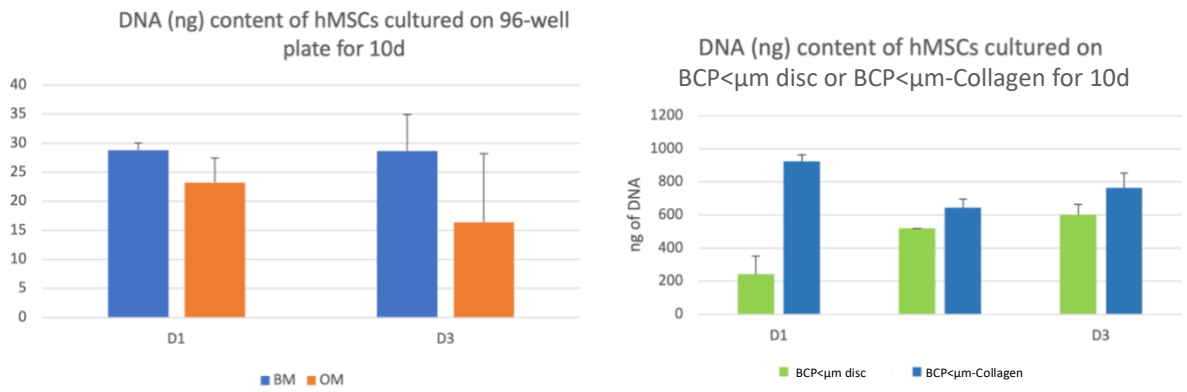

Figure 9. DNA quantification of hMSCs after 10 days of culture on BCP<µm discs or BCP<µm-

Collagen. After 10 days of culture on either BCP<µm discs (green) or BCP<µm-Collagen (dark blue), the DNA quantification was performed using CyQuant dsDNA kit and fluorescence was measured using Ex. 483 nm and Em. 530 nm filters. Negative controls (BM, dark blue) and positive controls (OM, orange) are also shown (left). Data are shown as mean ± standard deviation.

The quantification of ALP using Ex. 405 nm filter normalized for DNA revealed that hMSCs express twice the amount of ALP per cell when cultured on BCP<µm-Collagen compared to BCP<µm discs: 0.005 U/ng vs 0.0025 U/ng respectively (Figure 10 right). Moreover, hMSCs only differentiate in the presence of OM indicating a healthy state of the cells (Figure 10 left).

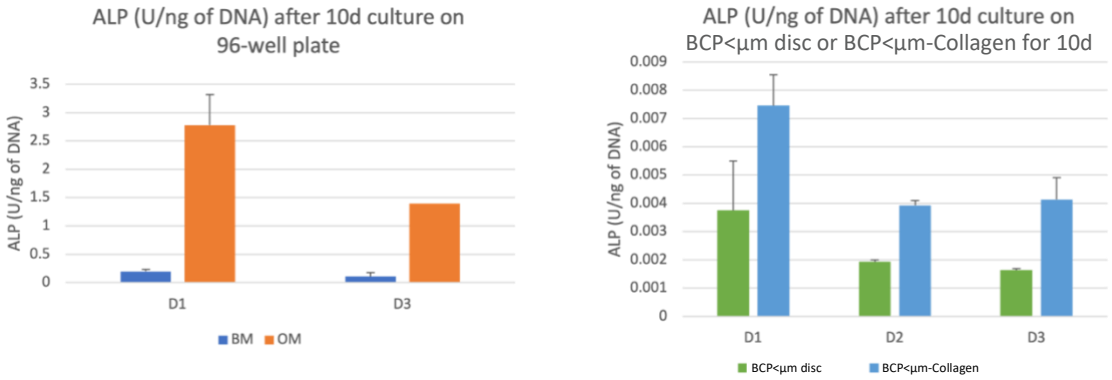

Figure 10. ALP quantification of hMSCs after 10 days of culture on BCP<µm discs or BCP<µm-Collagen. ALP content after 10 days of hMSC culture on BCP<µm discs (green) or BCP<µm-Collagen (light blue) was normalized for DNA to obtain ALP/ng of DNA (right). Negative controls (BM, dark blue) and positive controls (OM, orange) are also shown (left). Data are shown as mean ± standard deviation.

Discussion

In this study, a new *in vitro* platform consisting of BCP<µm discs was employed to study the osteostimulation of BCP<µm-Collagen. SEM analysis and Methylene blue staining of hMSCs cultured on the surface of BCP<µm discs for up to 10 days revealed hMSC attachment, proliferation, and progressive coverage of the disc. Moreover, hMSCs shifted from a flat morphology at 24 hours after seeding to an elongated one at 10 days which is typical of their osteogenic commitment. Hence, the discs showed suitability for cell culture and were further employed to compare hMSC metabolic activity, proliferation, and osteogenic differentiation between BCP<µm discs and the new device BCP<µm-Collagen. After culturing for 24 hours the hMSCs on the surface of BCP<µm discs or BCP<µm-Collagen in OM, a substantial increase in the metabolic activity was observed in BCP<µm-Collagen (2 out of 3 donors) which can be attributed to the presence of collagen as it greatly facilitates cell absorption and attachment to the material. In fact, collagen contains RGB domains which are easily recognized and bound by the cells, and they could be the underlying cause of an easy attachment by the hMSCs. Although the metabolic activity of hMSCs was higher in BCP<µm-Collagen within the first 24 hours of culture, no difference in DNA content was observed after 10 days indicating that cell proliferation over time is similar between BCP<µm discs and BCP<µm-Collagen (given an equal volume). Although total cell content did not differ between the two materials, ALP content normalized for DNA was higher (2-folds) in BCP<µm-Collagen compared to BCP<µm discs, hence hMSC osteogenic differentiation was more effective when employing BCP<µm-Collagen. Moreover, hMSCs only differentiated in the presence of OM when cultured on 96-well plates indicating a healthy state of the cells. Of note, a significant reduction in ALP expression per cell was observed when the hMSCs were cultured on the materials compared to the control wells. This could indicate different kinetics of

differentiation when the cells are cultured on the materials compared to culture plates, or a difficult release of ALP into the media. However, calcium phosphates like BCP<µm can easily bind proteins and, although the presence of FBS should block this process, it cannot be excluded that part of the ALP produced by the cells was not released into the media. For this reason, ALP staining should be performed on the disc. Nevertheless, BCP<µm-Collagen showed potential osteoconductive properties, and greatly favours cell attachment and metabolic activity, hence BCP<µm-Collagen is a promising strategy to allow easy implantation of BCP<µm granules and harnessing bone-forming properties. Of note, in all experiments, D1 did not efficiently attach to BCP<µm discs, whereas it did attach and proliferate on BCP<µm-Collagen. Hence, the presence of collagen might rescue cell attachment when it fails to do so on BCP<µm. Limitations of this study include few time points investigated (24 hours and 10 days only), and few osteogenic markers evaluated (ALP only).

Conclusion

In conclusion, BCP<µm discs are a promising predicate device to compare hMSC attachment, proliferation, and osteogenic differentiation with newer devices (i.e., BCP<µm-Collagen). hMSCs cultured on the surface of the BCP<µm discs showed attachment, morphological shift towards elongated shape, and progressive coverage of the surface of the disc over time. After comparing hMSC metabolic activity, proliferation, and differentiation when cultured on the surface of either BCP<µm discs or BCP<µm-Collagen, an increased metabolic activity, and osteogenic differentiation per cell were observed in BCP<µm-Collagen, with no differences in DNA content between the materials after 10 days of culture. Hence, BCP<µm-Collagen is a promising bone-filling biomaterial harnessing osteostimulating properties.

Future experiments

Alamar Blue assay should be performed also at 10 days to identify an eventual increase in metabolic activity at later time points. DNA quantification should also be performed at 24 hours to confirm that the increase in metabolic activity in BCP<µm-Collagen corresponds to more metabolically active hMSCs or increased hMSCs attaching to the material. A PCR on genes characteristic of hMSC osteogenic differentiation or mineralization assays should also be performed to confirm an increased end-stage osteogenic differentiation on BCP<µm-Collagen. Comparing the performance of BCP<µm-Collagen to BCP<µm granules is also recommended.

Laymen summary

The recent discovery of the close relationship between the immune system and bone homeostasis has brought osteoimmunology to the forefront of bone regeneration. Bone fillers capable of inducing the formation of new bone by stimulating immune cells to release soluble factors that affect hMSC recruitment and differentiation towards osteoblasts are the most promising bone grafts for predictable bone regeneration. Among them, BCP<µm, a biphasic calcium phosphate with submicron needle-shaped surface topography, showed effective bone formation both in vitro and in vivo. This study aimed to evaluate whether the addition of a collagen matrix to BCP<µm granules positively affects the differentiation of hMSCs towards osteoblasts. To do so, discs of BCP<µm granules (referred at BCP<µm discs) were produced and investigated for hMSC attachment and proliferation. When hMSCs are cultured on the surface of BCP<µm discs for 10 days, a progressive coverage of the surface of the discs over time was observed, together with a shift from a flat morphology at 24 hours after seeding to an elongated one at 10 days. These results suggest that hMSCs attach, proliferate, and differentiate on BCP<µm discs. Once the suitability of BCP<µm discs for cell culture was determined, the potential of the BCP<µm-Collagen to promote hMSC metabolic activity and differentiation towards osteoblasts was compared to BCP<µm discs. The Alamar blue, ALP, and DNA assays were performed on the materials to test our hypothesis. The Alamar blue assay on hMSCs

cultured on the surface of the BCP<µm discs or BCP<µm-Collagen for 24 hours showed up to 20-fold increase in metabolic activity. Moreover, ALP and DNA quantification after 10 days of stimulation with OM showed up to 2-fold increase in ALP production per cell. No differences in DNA content of hMSCs were observed between BCP<µm discs or BCP<µm-Collagen after 10 days of culture. These results suggest that collagen substantially favours cell attachment and metabolic activity within the first 24 hours of hMSC culture. However, the total number of hMSC did not differ at 10 days. Moreover, an increase in osteogenic differentiation towards osteoblast was reported in BCP<µm-Collagen. Hence, the addition of Collagen to BCP<µm granules does not hinder Granules' function and BCP<µm-Collagen represents a promising bone filler with osteostimulating properties.

PART 2 – Pro-angiogenic properties of collagen-elastin sponges

Abstract

Blood vessels serve as a structure around which bone regeneration occurs, and newly formed blood vessels promote the local accumulation of soluble factors that promote the recruitment and differentiation of bone precursor cells towards osteoblasts. Elastin is a key component of elastic fibers, and in vitro tests showed that elastin promotes the migration and differentiation of HUVECs, which are the most employed cell types to study angiogenesis. To discover whether the addition of elastin to collagen matrices augments their pro-angiogenic properties resulting in more predictable bone regeneration, we cultured HUVECs for 16 hours on the surface of reconstituted collagen or collagen-elastin sponges to assess whether elastin positively regulated network formation. Then, an Alamar blue assay was performed on HUVECs cultured directly onto the surface of the lyophilized sponges to assess whether elastin promotes a higher metabolic activity in HUVECs. The network analysis revealed an increase in the number of junctions, total length, and total branching length when HUVECs were cultured in the presence of elastin compared to pure collagen gels. In particular, a 100% increase in the number of junctions, a 40% increase in total length, and an 82% increase in total branching length were observed in the presence of elastin. Finally, the metabolic assays revealed a 2-fold increase in reduction capability when HUVECs were cultured on the surface of collagen-elastin sponges compared to pure collagen sponges. In conclusion, elastin positively regulates network formation and metabolic activity in HUVECs, hence it represents a promising candidate to confer angiogenic properties to bone implants aiming to augment their overall osteoinductivity.

Definitions and Abbreviations

HUVECs	Human Umbilical Vein Endothelial Cells
EDPs	Elastin-Derived Peptides

Introduction

The link between bone and blood vessel formation was firstly documented in the early 1700s and it is now largely recognized. Blood vessels serve as a structure around which bone development occurs, and the formation of microvasculature is essential for the homeostasis and regeneration of the bone tissue [10]. In addition, blood vessels are a source of paracrine factors which promote the recruitment and differentiation of bone precursor cells. In fact, the natural inflammatory response resulting from injury or graft implantation up-regulates vascularization due to the soluble factors released by several cell types which in turn favours tissue regeneration [19].

Elastin is the primary component of elastic fibers which are a major part of elastic tissues including arteries, lungs, cartilage, skin, breast, and ligaments. Upon injury, elastin fibers are not readily replaced due to the complexity of their biosynthesis halting tissue regeneration. Interestingly, elastin peptides and fragments released upon elastin degradation, called elastin-derived peptides (EDPs), have shown a wide range of biological activities underlying tissue regeneration and

vascularization [20,21]. By culturing HUVECs, the precursors of endothelial cells, with EDPs in Matrigel, an increased proliferation, migration, and pseudo-tube formation were observed [20]. In particular, increased levels of MT1-MMP were noted which in turn may lead to an increased expression of VEGF by trans-activating VEGFR-1 [22]. Hence, the overexpression of MT1-MMP by EDPs may play an important role in angiogenesis. Moreover, EDPs were capable of promoting the up-regulation of bone proteins by binding elastin laminin receptors (ELRs) on smooth muscle cells (SMCs) resulting in vascular calcification [23]. Interestingly, the effects of elastin-based biomaterials have also been investigated in MSCs [24]. Porcine MSCs cultured in the presence of recombinant elastin-like polypeptides showed increased attachment and proliferation in vitro, and an increased osteogenic differentiation was also noted when bmMSCs were cultured with silk-tropoelastin scaffolds [24]. Hence, elastin-based biomaterials can promote a wide range of bioactive processes which may positively regulate bone formation. Therefore, incorporating BCP<µm granules in a collagen-elastin matrix might represent a new valid strategy to promote bone formation and vascularization simultaneously.

As described by others, HUVECs are the most employed cell types for the in vitro assessment of angiogenesis, and they are usually evaluated for proliferation, migration, and differentiation [25]. Hence, we investigated the response of HUVECs to pure collagen or collagen-elastin sponges in terms of metabolic activity and tube formation.

Materials and Equipment

Generation of collagen and collagen-elastin sponges for tube formation assay

To visualize the network formed by HUVECs in the presence of collagen or collagen-elastin, the sponges provided by MedSkin™ were converted into gels following the protocol described by Rayan, N. et al., 2007 [26]. In brief, the sponges were blended at low temperature (< 9 °C) to avoid elastin or collagen degradation in 0.02 N cold acetic acid diluted in medical-grade water, and the obtained viscous solutions were de-aerated for 15 minutes. After that, the solutions were poured into 16 cm strips of SPECTRAPOR dialysis membrane tubing (Thermo Scientific™ - 11425939) and placed into a 4-liter baker with cold 0.02N acetic acid for 1h at 4 °C. Then, the dialysis tubes containing collagen or collagen-elastin were placed into a 1% chloroform solution diluted in ultrapure water for 1h at 4 °C. Finally, the dialysis bags were placed into 4 L of sterile 0.0 2 N acetic acid at 4 °C for 1 week with the acetic acid replaced every 2 days. Once sterile, the content of the tubes was poured into sterile 100 mL jars and stored at 4 °C until use. Before use, the solutions were neutralized using sterile 0.1 N NaOH in water until pH 6.5-7 which leads to jellification at 37 °C.

Preparation of IBIDI µ-slide for tube formation assay

IBIDI µ-slide for angiogenic assays (IBIDI™ – 81506) were placed at -20 °C for 24 hours before starting the experiment. Matrigel was thawed at 4 °C overnight, and 10 µL were pipetted into the wells of the slides. The slides were then incubated at 37 °C for 30 mins to allow gel formation. After that, 50 µL of neutralized collagen or collagen-elastin solutions were pipetted into the wells and further incubated for 24 hours to allow the sedimentation of collagen or collagen-elastin onto the Matrigel.

Tube formation assay of HUVECs cultured or collagen or collagen-elastin gels

HUVECs from pooled donors between P3 and P5 were thawed and seeded at $3x10^3$cells/cm^2 in 0.1% gelatin-coated T175 flasks in EGM-2 BulletKit (Lonza™ - CC-3162) consisting of EBM-2 supplemented with heparin, hEGF, Hydrocortisone, Ascorbic Acid, GA-1000 (Gentamicin, Amphotericin B), 10% (v/v) heat-inactivated FBS (Fetal Bovine Serum), VEGF, hFGF-B, and R^3-IGF-1. The flasks were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 until HUVECs reached 70% confluency. Once ready-to-use, HUVECs were detached, centrifuged at 200 g, counted, and adjusted to $1,2x10^5$ cells/mL in EBM-2 supplemented with either no FBS (negative control) or 5% (v/v) heat-inactivated FBS. After that, the collagen and collagen-elastin solutions were replaced with

50 µL of cell suspension (6x10^3 HUVECs/well). The slides were then incubated for 16 hours at 37°C in a humidified atmosphere containing 5% CO_2 to allow tube formation. Imaging was performed using an optical microscope at 4x magnification with the diaphragm almost entirely closed to highlight the contrast between the gel and the HUVECs. The quantification was performed using AngioQuant macro in ImageJ. An overview of the experiment is shown below.

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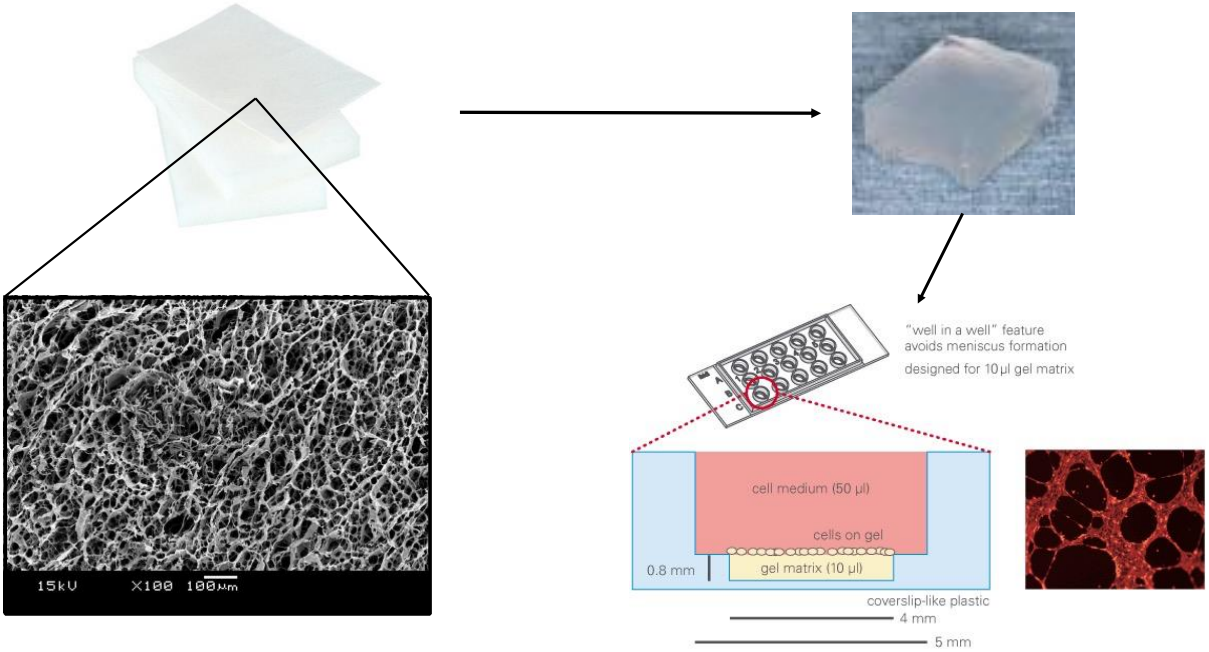

Figure 1. Overview of the experiment.

Metabolic activity of HUVECs cultured on collagen or collagen-elastin sponges

The sterile collagen and collagen-elastin sponges were provided by MedSkin™ and used as received. Pieces of the sponges were cut into square shapes of roughly 0.2 g and pre-incubated in EBM-2 (Lonza™ - CC-3162) supplemented with 5% (v/v) heat-inactivated FBS for at least 4 hours in 25-well plates for low cell attachment. After that, HUVECs were adjusted to 1x10^6 cells/mL and 150 µL of suspension was seeded onto the sponges. After 2h incubation to allow cell attachment, 2 mL of EBM-2 supplemented with 5% (v/v) heat-inactivated FBS was added to the cells and the plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. HUVECs seeded in 96-well plates pre-coated with 0.1% gelatin in EBM-2 supplemented with 5% FBS was used as a positive control. After 24 hours incubation, the media was aspirated from the wells, the Alamar blue was diluted 10 times in EBM-2 supplemented with 5% FBS, and 1mL was added to the wells. The plates were again incubated for 4 hours. After the incubation, 100 µL of media per group was collected and stored at 4 °C protected from light until analysed. The analysis was performed with Ex. 544 nm and Em. 590 nm filters. An overview of the experiment is shown below (Figure 2).

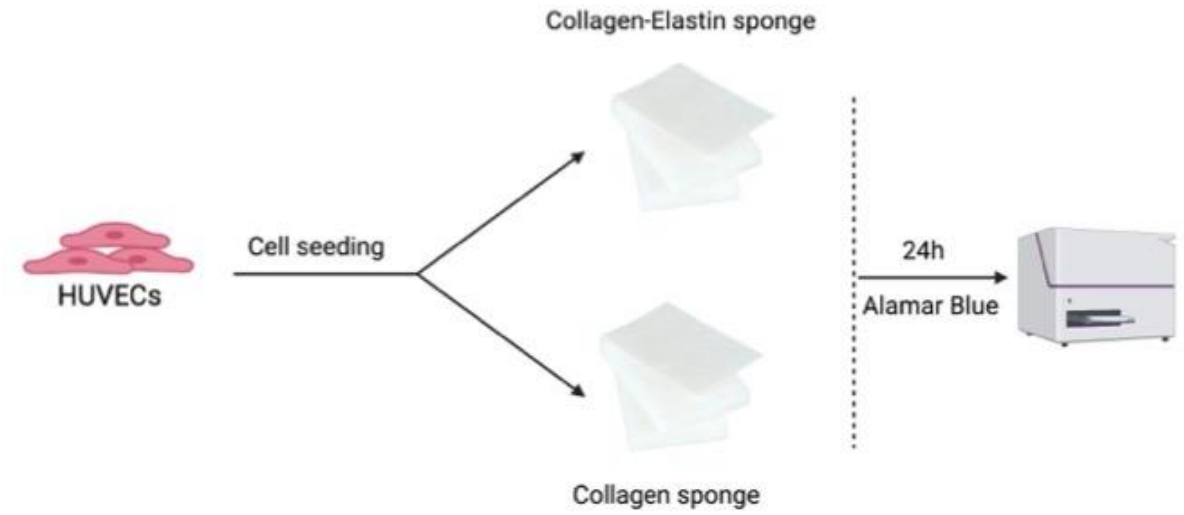

Figure 2. Overview of the experiment to evaluate HUVEC metabolic activity on collagen or collagen-elastin sponges.

Results

Tube formation analysis of HUVECs on reconstituted collagen or collagen-elastin sponges

Representative images of HUVECs cultured in the presence of collagen (left) or collagen-elastin (right) in either 0% FBS (top) or 5% FBS (bottom) are shown in Figure 3. The red dots represent the nodes.

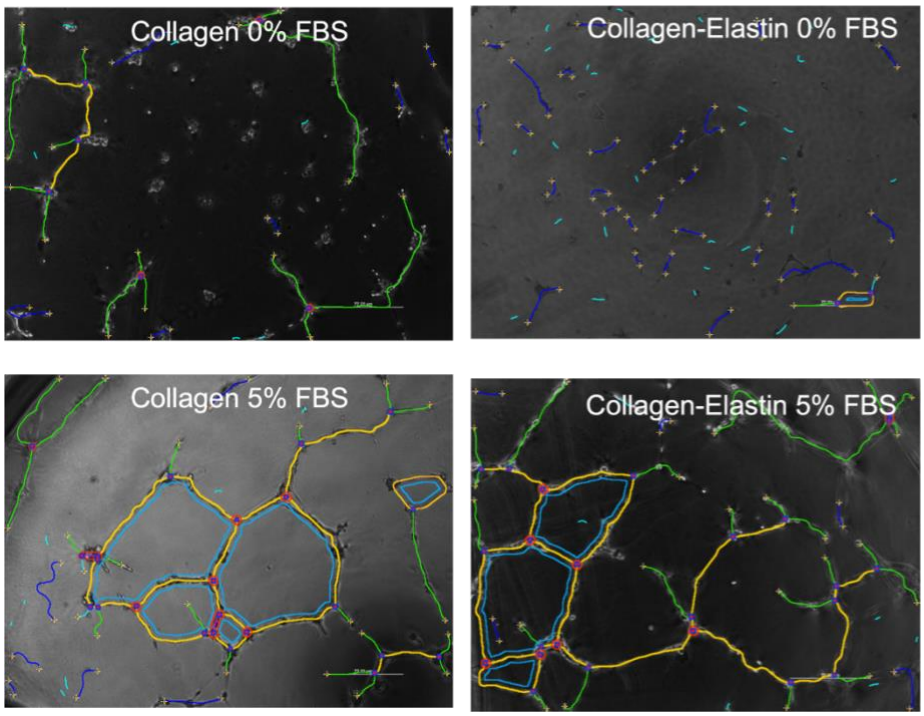

Figure 3. Imaging and identification of HUVEC network formation through AngioQuant macro in ImageJ. HUVECs cultured in the presence of collagen (left) or collagen-elastin (right) in either 0% FBS (top) or 5% FBS (bottom) are shown.

Once several images per well had been acquired, the pictures were loaded into ImageJ and analysed using AngioQuant macro in ImageJ. As readouts, the number of junctions, total length, and total branching length were chosen. The analysis showed an increase in total branching length (1850 vs 1016, p-value: 0.02), number of junctions (10 vs 5, p-value: 0.04), and total length (2475 vs 1727, p-value: 0.03) when HUVECs were cultured on collagen-elastin sponges compared to collagen sponges with 5% (v/v) heat-inactivated FBS as shown in Figure 4. As expected, no network formation is observed when FBS is not added to the media (negative controls).

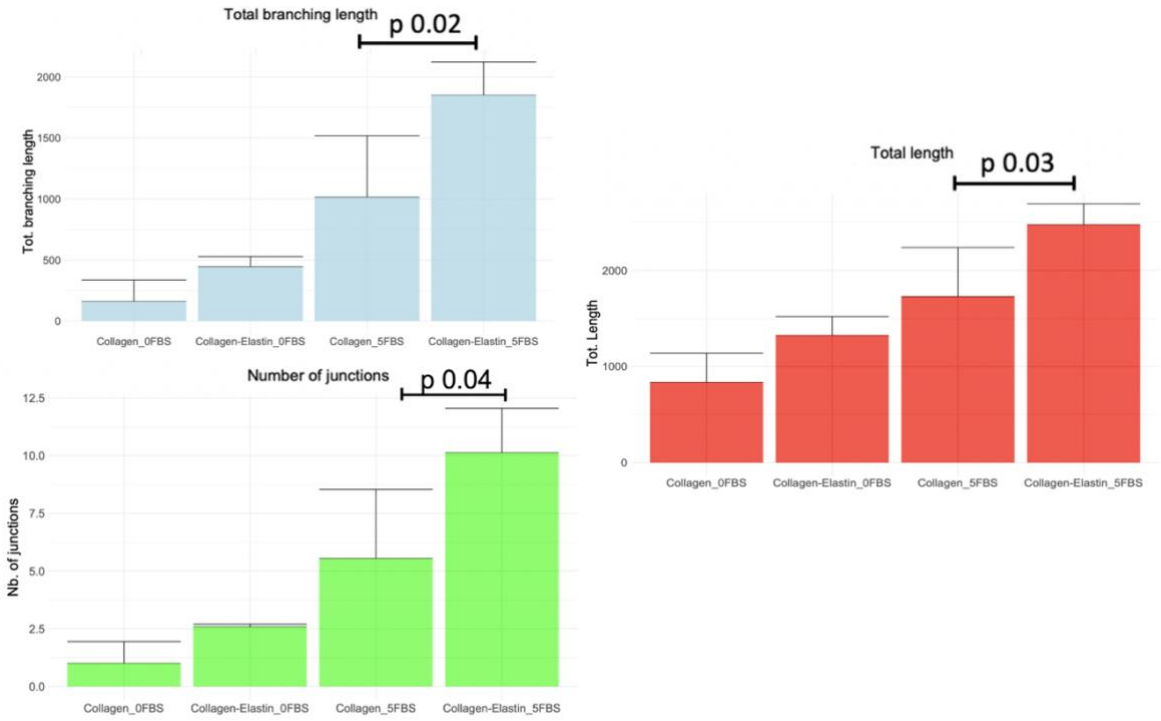

Figure 2. Quantification of network performance. The quantification of total branching length (blue), number of junctions (green), and total length (red) of the network was performed using AngioQuant macro in ImageJ. Data are shown as mean ± standard deviation. Statistical analysis used: two-sample t-test with alpha=0.05.

Metabolic assay on collagen and collagen-elastin sponges

After culturing the HUVECs on the surface of collagen or collagen-elastin sponges for 4 hours in the presence of Alamar blue, the analysis revealed a 2-fold increase in reduction capability when HUVECs were cultured on the surface of collagen-elastin sponges compared to pure collagen sponges (Figure 3). No difference between HUVECs cultured on collagen-elastin sponges and the positive control gelatin-coated tissue culture plates were noted.

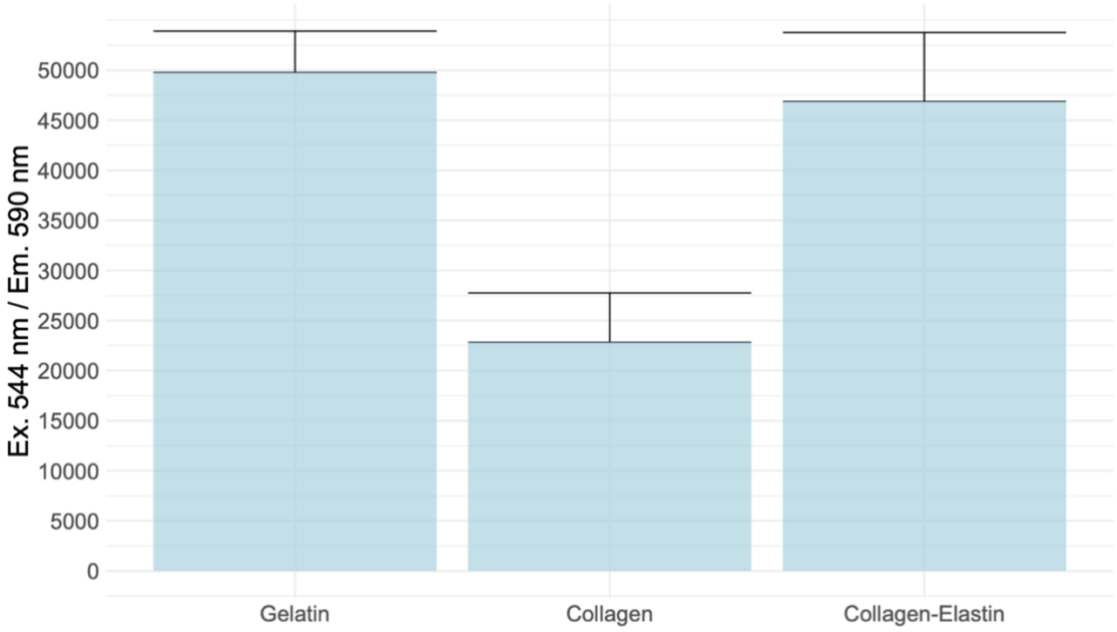

Figure 3. Quantification of the metabolic activity of HUVECs. The metabolic activity of HUVECs cultured for 24 hours on either gelatin-coated 96-well plates (positive control), pure collagen sponges, or collagen-elastin sponges was quantified using fluorescence intensity at Ex. 544 nm and Em. 590 nm**.** Data are shown as mean ± standard deviation. The experiment was performed in triplicates with HUVECs from pooled donors.

Discussion

Angiogenesis has been shown to positively regulate bone formation since newly formed blood vessels promote the local accumulation of pro-osteogenic factors. Among the different assays developed to study angiogenesis in vitro, network formation, differentiation, and migration assay are the most employed. In several settings, elastin and its peptides have been shown to promote angiogenesis in HUVECs in vitro. Driven by these evidences, a new formulation of collagen-elastin has been developed aiming to embed BCP<µm granules in it. By doing so, it would be possible to simultaneously stimulate bone and blood vessel formation. In this study, we investigated whether the addition of elastin to a collagen matrix positively affects network formation and metabolic activity in HUVECs, important biological processes underlying bone regeneration. To do so, we cultured HUVECs on reconstituted collagen and collagen-elastin sponges to assess network formation. HUVECs cultured in the presence of collagen-elastin for 16 hours showed increased performances of the network in terms of number of junctions, total length, and total branching length compared to pure collagen. Moreover, in the absence of FBS, no network is formed indicating the validity of this testing platform. To confirm that elastin positively regulates the metabolic activity, an Alamar blue assay was performed on the collagen or collagen-elastin sponges in a non-disruptive manner. After culturing HUVECs on the surface of the sponges for 24 hours, the metabolic assay revealed increased metabolic activity of HUVECs on the surface of the collagen-elastin sponges (comparable to the positive control 96-well plates coated with 0.1% gelatin), and a reduced metabolic activity was noted when HUVECs were cultured on the surface of pure collagen sponges. This indicates that better network performance in the presence of elastin might correlate with the increased metabolic activity in HUVECs. However, Matrigel was used to create a gel basement to allow tube formation, and it would be more relevant if collagen or collagen-elastin gel would be evaluated alone. Other limitations of this study include the missing evaluation of pro-angiogenic growth factors and cytokines released in the media, and the

assessment of HUVEC migration in the presence of elastin, an important process for early angiogenesis.

Conclusion

In this study, we provided evidence that collagen-elastin sponges positively regulate early angiogenesis in HUVECs as evaluated by network formation and metabolic assays. In both assays, collagen-elastin outperforms collagen in up-regulating HUVEC reduction capability of Alamar blue indicating a more active state of the cells, and their ability to form a network, a key step underlying angiogenesis. However, to draw solid conclusions, more investigations should be carried out to confirm the better performances of collagen-elastin in inducing HUVEC differentiation and production of pro-angiogenic factors.

Future experiments

ELISA on conditioned media produced by HUVECs on collagen or collagen-elastin sponges should be performed to confirm an eventual increase in pro-angiogenic factors (i.e., VEGF) released by the HUVECs. As migration is also an important process for tube formation, the migrating potential of HUVECs should also be evaluated in a wound-healing assay. Assessing an increased expression of proteins in HUVECs when cultured on collagen-elastin sponges (i.e., MMPs) is also recommended. Finally, the evaluation of the in vitro performances of the sponges with BCP<µm granules embedded in them, together with in vivo assessments, should also be performed.

Laymen Summary

As described by others, vascularization and bone formation are coupled processes. Through the formation of new blood vessels, soluble factors can reach the damaged site and promote the recruitment and differentiation of bone precursor cells. Hence, the addition of pro-angiogenic factors to bone biomaterials represent a vigorous strategy to enhance their osteoinductive properties. In this study, we investigated whether the addition of elastin to a lyophilized collagen sponge improves the ability of HUVECs to form a network with applications for bone grafts. To do so, the lyophilized sponges of collagen or collagen-elastin were reconstituted and used to image the network formed by HUVECs seeded on their surface for 16 hours. The analysis revealed an increase in the number of junctions, total length, and total branching length when HUVECs were cultured in the presence of elastin compared to pure collagen. In particular, a 100% increase in the number of junctions, a 40% increase in total length, and an 82% increase in total branching length were observed in the presence of elastin. Finally, an Alamar blue assay was performed on HUVECs cultured directly onto the surface of the lyophilized sponges to assess whether elastin promotes a higher metabolic activity in HUVECs. The analysis revealed a 2-fold increase in reduction capability when HUVECs were cultured on the surface of collagen-elastin sponges compared to pure collagen sponges. No difference between HUVECs cultured on collagen-elastin sponges and the positive control gelatin-coated tissue culture plates were noted. In conclusion, elastin positively regulates network formation and metabolic activity in HUVECs, hence it represents a promising candidate to confer angiogenic properties to bone implants aiming to augment their overall osteoinductivity.

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