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van Esterik, F.A.S.

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CHAPTER 5

HYPOXIA DECREASES OSTEOGENIC DIFFERENTIATION POTENTIAL BUT ENHANCES VASCULOGENIC DIFFERENTIATION POTENTIAL OF HUMAN ADIPOSE STEM CELLS ON BIPHASIC CALCIUM PHOSPHATE SCAFFOLDS IN FIBRIN GELS

Fransisca A.S. van Esterik\textsuperscript{1,2}, Jolanda M.A. Hogervorst\textsuperscript{1}, Pieter Koolwijk\textsuperscript{3}, Jenneke Klein-Nulend\textsuperscript{1}

\textsuperscript{1} Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit Amsterdam, MOVE Research Institute Amsterdam, Amsterdam, The Netherlands

\textsuperscript{2} Department of Dental Materials Science, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit Amsterdam, MOVE Research Institute Amsterdam, Amsterdam, The Netherlands

\textsuperscript{3} Department of Physiology, Institute of Cardiovascular Research, VU University Medical Center

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ABSTRACT
In bone tissue engineering, biphasic calcium phosphate (BCP) with different hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) ratios can be combined with regeneration-competent stem cells, and bioscaffolds like fibrin that trigger neovascularization. We have shown recently that human adipose stem cells (hASCs) on BCP with a HA/β-TCP ratio of 20/80 (BCP20/80) incorporated in fibrin gel show enhanced osteogenic and/or vasculogenic differentiation potential compared to BCP with a HA/β-TCP ratio of 60/40 (BCP60/40) in vitro. A hypoxic microenvironment for the cells might occur in these hASC-seeded BCP-based composites after implantation in vivo. This limits oxygen supply which is essential for successful bone tissue engineering. Here we aimed to test the effect of hypoxia on osteogenic and/or vasculogenic differentiation of hASC-seeded BCP60/40 or BCP20/80 scaffolds in fibrin gels as well as fibrin gel degradation. BCP60/40-based composites and BCP20/80-based composites were cultured under normoxia (20% CO₂) or hypoxia (1% CO₂) for 12 days, and total DNA content, alkaline phosphatase activity, nitric oxide production, vascular endothelial growth factor production, and fibrin gel degradation were assessed during culture. Hypoxia similarly decreased total DNA content at day 7 (1.3-fold) and 12 (1.2-fold) in both composites. It also decreased alkaline phosphatase activity at day 5 (5.0 and 4.7-fold), day 7 (4.5 and 6-fold), and 12 (7.1 and 6.8-fold) in BCP60/40-based composites and BCP20/80-based composites. Hypoxia similarly increased vascular endothelial growth factor production at day 5 (3.9-fold) and day 7 (2.6-fold) by both BCP-based composites. Hypoxia did not affect nitric oxide production and fibrin gel degradation in both BCP-based composites at all time points. In conclusion, hypoxia decreased osteogenic differentiation but enhanced vasculogenic differentiation of cultured BCP-based composites. These in vitro results implicate that cell-seeded BCP-based composites implanted in vivo, might enhance vascular endothelial growth factor production resulting in sprouting and tube formation leading to increased survival of cells in these composites, and stimulation of bone formation.

Key words: Adipose stem cells, Biphasic calcium phosphate, Hypoxia, Osteogenic differentiation, Vasculogenic differentiation
INTRODUCTION
Bone tissue engineering is based on combinations of scaffolds, cells (including stem cells), and mechanical and/or chemical stimuli. For clinical bone augmentation, such as maxillary sinus floor elevation, autologous bone is the golden standard. An alternative to the golden standard is biphasic calcium phosphate (BCP), which is composed of hydroxyapatite (HA) and \( \beta \)-tricalcium phosphate (\( \beta \)-TCP), and its chemical composition resembles the inorganic part of the natural bone matrix. In bone tissue engineering, BCP with different HA/\( \beta \)-TCP ratios can be combined with regeneration-competent stem cells, such as freshly isolated autologous stromal vascular fraction of adipose tissue and adipose tissue-derived mesenchymal stem cells (ASCs). These cell-based bone constructs have been successfully used for bone augmentation in e.g. maxillary sinus floor elevation.

After implantation of cell-seeded BCP constructs in vivo, the disrupted blood vessels at the implant site may rapidly lead to a hypoxic microenvironment until neovascularization occurs. In vitro simulation of this microenvironment might offer a realistic scenario of how cell-seeded BCP constructs may preserve their features and achieve their function in vivo. Hypoxia is an important feature of the cellular microenvironment which maintains stem cells in an undifferentiated state, but no consensus has been reached about the influence on DNA repair. Understanding the role of a hypoxic microenvironment is essential for the development of successful tissue engineering approaches including bone regeneration, since the physiological oxygen concentration in bone can be as low as 1.3%. The effects of hypoxia on human ASC (hASC) expansion and differentiation are contrasting and no consensus has been reached. Although the effect of hypoxia on ASC differentiation is strongly dependent on cultivation conditions, the differentiation of ASCs towards the osteogenic lineage is likely to be inhibited by hypoxia. On the other hand, the combination of hypoxia and osteogenic stimuli accelerates ASC differentiation and mineralized matrix production in vitro. Implantation of BCP with a HA/\( \beta \)-TCP ratio of 20/80 (BCP20/80) in patients undergoing unilateral maxillary sinus floor elevation resulted in more ingrowth of newly formed bone tissue at the most cranial side compared to patients treated with BCP60/40 (unpublished data), but a correlation with bone formation has not yet been established. Cell-seeded BCP60/40 implanted in patients undergoing bilateral maxillary sinus floor elevation also resulted in more active bone formation at the cranial side compared to the middle part of the grafted area, which might be correlated to increased blood vessel formation.

For clinical application of these cell-based bone constructs, a functional blood vessel network is necessary to allow nutrient diffusion and waste removal, which is crucial for cell recruitment, survival, proliferation, differentiation, and tissue remodeling. Osteogenesis and angiogenesis are tightly coupled processes, and vascular development needs to be induced before osteogenesis can take place. This complexity has been a major challenge for
engineering viable and functional bone constructs.\textsuperscript{14,15} Cell-based bone constructs might be combined with bioscaffolds to enhance vascular development.\textsuperscript{16} hASCs seeded on BCP20/80 incorporated in a fibrin bioscaffold show enhanced osteogenic and vasculogenic differentiation potential compared to BCP60/40.\textsuperscript{17}

Understanding the effect of hypoxia on the osteogenic and/or vasculogenic differentiation of composites consisting of hASC-seeded BCP60/40 or BCP20/80 scaffolds incorporated in fibrin gel is essential for bone tissue engineering. Therefore, we aimed to test the effect of hypoxia on osteogenic and/or vasculogenic differentiation potential as well as degradation of BCP60/40-based composites and BCP20/80-composites. We hypothesized that hypoxia would decrease osteogenic differentiation, and enhance vasculogenic differentiation potential in both composites. hASCs were seeded on BCP60/40 or BCP20/80 scaffolds, with similar pore size and porosity, and then incorporated in fibrin gel, and cultured for 12 days. Total DNA content, osteogenic and vasculogenic differentiation potential, and fibrin degradation were assessed up to 12 days. In vitro tube formation was determined at day 19. This study reports the effect of hypoxia on the osteogenic and/or vasculogenic differentiation potential of BCP20/80 and BCP60/40-based composites, and demonstrates that hypoxia decreases osteogenic differentiation potential and enhances vasculogenic differentiation potential in both BCP-based composites.

**MATERIALS AND METHODS**

**Biphasic calcium phosphate scaffolds**
Two different calcium phosphate scaffolds were used: (1) Straumann® BoneCeramic 60/40 (Institut Straumann AG, Basel, Switzerland), a porous BCP scaffold composed of 60% HA and 40% β-TCP (BCP60/40), and (2) Straumann® BoneCeramic 20/80 (Institut Straumann AG), a porous BCP scaffold composed of 20% HA and 80% β-TCP (BCP20/80). To avoid differences in osteogenic and vasculogenic potential of hASCs seeded on BCP60/40 or BCP20/80 incorporated in fibrin gels caused by the BCP fabrication process, the two different BCPs were produced by the same company, and had similar pore size and porosity.\textsuperscript{17}

**Donors**
Subcutaneous adipose tissue was harvested from residues of abdominal wall resections of five healthy female donors (aged 33, 40, 47, 50, and 54), who underwent elective abdominal wall correction at the Tergooi Hospital Hilversum and a clinic in Bilthoven, The Netherlands. The Ethical Review Board of the VU Medical Center, Amsterdam, The Netherlands, approved the protocol. Informed consent was obtained from all patients.
Isolation and culture hASCs

hASCs were isolated from the resection material from above mentioned donors as described with minor modifications. In brief, adipose tissue was cut into small pieces, and enzymatically digested with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 45 min at 37°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Roche Diagnostics GmbH) under continuous stirring. Ficoll® density-centrifugation step (Lymphoprep™; Axis-Shield, Oslo, Norway; 1000xg for 20 min, ρ=1.077 g/ml Ficoll®, osmolarity 280±15 mOsm) was performed to remove remaining erythrocytes from the stromal vascular fraction. The resulting stromal vascular fraction pellet containing hASCs was resuspended in Dulbecco’s modified Eagle’s medium (Life Technologies™ Europe BV, Bleiswijk, The Netherlands), counted, frozen and stored in liquid nitrogen until further use. Heterogeneity studies including cell characterization and multipotent differentiation potential of these cells have been reported previously by our group.18 Cryopreserved stromal vascular fraction-containing cell suspensions were pooled and cultured in α-Minimum Essential medium (α-MEM; Life Technologies™, Waltham, MA, USA) with 5% platelet lysate (see below), 100 U/ml penicillin (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Sigma-Aldrich Biochemie GmbH), and 10 IU/ml heparin (LEO Pharma A/S, Ballerup, Denmark) to prevent coagulation at 37°C in a humidified atmosphere with 5% CO₂. The medium was refreshed three times/week. After reaching confluency, cells were harvested with 0.25% trypsin (LifeTechnologies™) and 0.1% ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) in PBS at 37°C, replated, cultured until passage 2 (P2), and stored in liquid nitrogen until further use. Cryopreserved pooled hASCs-containing cell suspensions were thawed and seeded at 0.5x10⁵ cells per T-225 culture flask (Greiner Bio-One, Kremsmuenster, Austria) in α-MEM with 2% platelet lysate, antibiotics, and 10 IU/ml heparin. Cells were cultured until P3-P4, and used for composites preparation (see below).

Platelet-lysate

Pooled platelet products from five donors were obtained from the Bloodbank Sanquin (Sanquin, Amsterdam, The Netherlands). Platelet lysate was obtained by lysing the platelets through temperature-shock by freezing at -80°C, thawing, and centrifugation at 600xg for 10 min to eliminate remaining platelet fragments. The supernatant was added at 2% (v/v) to the medium.

Fibrin gel

Human fibrinogen plasminogen-depleted protein (Enzyme Research Laboratories, South Bend, IN, USA) was dissolved in Medium 199 (M199; Life Technologies™) with antibiotics at 37°C for 1 h. Solubilized fibrinogen was filtered through a 0.2 µm filter (Millipore, Amsterdam,
The Netherlands), and the concentration measured with a Synergy HT® spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). To prepare fibrin gel, 2 mg/ml fibrinogen solution was polymerized with 1.0 IU/ml bovine α-thrombin (Enzyme Research Laboratories) in a buffer containing 50 mM sodium citrate, 0.2 M sodium chloride, and 0.1% polyethylene glycol-8000, for 1 h at room temperature followed by 1 h at 37ºC, and used for preparation of BCP60/40 and BCP20/80-based composites (see below), as well as for fibrin gel coating of polystyrene 48-well culture plates (Cellstar, Greiner Bio One International GmbH, Frickenhausen, Germany) used for culture of these composites.

**Composites preparation and culture**

Cultured hASCs were washed three times with PBS to remove platelet lysate, and either seeded on BCP60/40 or BCP20/80 scaffolds and then incorporated in fibrin gels to prepare BCP60/40 and BCP20/80-based composites. Twenty-five to 30 mg BCP60/40 or BCP20/80 was hydrated in PBS for 30 min. After PBS removal, 1x10^5 ASCs in 100 µl α-MEM were allowed to attach for 30 min at room temperature. Cell-seeded BCP scaffolds were embedded in fibrin gels and placed on fibrin gel-coated plates.

BCP60/40-based composites and BCP20/80-based composites were cultured in α-MEM (Life Technologies™) with 2% platelet lysate, antibiotics, 50 µg/ml 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and 10 IU/ml heparin for 12 days at 37ºC, at normoxic culture conditions in a humidified atmosphere of 20% O₂ and 5% CO₂ or at hypoxic culture conditions (see below) in a humidified atmosphere of 1% O₂ and 5% CO₂. Medium was refreshed after 7 days.

**Hypoxic cell culture**

Hypoxic cell culture conditions were maintained inside a custom designed hypoxic workstation (TCPS, Rotselaar, Belgium), with a O₂ (via injection of N₂), and CO₂ controlled, humidified incubator (Sanyo, Ettenleur, The Netherlands), placed inside a T4 glovebox (Jacomex, Dagneux, France) equipped with an O2X1 oxygen transmitter (GE Infrastructure Sensing, Inc., Billerica, MA, USA). The oxygen concentration inside the incubator was continuously monitored with an internal zirconia sensor and periodically checked with O₂ test tubes (Drager Safety, Zoetermeer, The Netherlands). To prevent reoxygenation during hypoxic culture, all media were pre-incubated for 3 h before use.

**Total DNA content in composites**

Total DNA content was assessed in BCP60/40-based composites and BCP20/80-based composites at day 1, 5, 7, and 12 by using alamarBlue® fluorescent assay (Invitrogen, Frederick, MD, USA), according to the manufacturer’s instructions. We found a linear relationship between alamarBlue® fluorescence and DNA content (data not shown).
Fluorescence was read in medium samples at 530 nm with a Synergy HT® spectrophotometer.

**Alkaline phosphatase activity**
Alkaline phosphatase (ALP) activity was measured to assess the osteoblastic phenotype of hASCs in BCP60/40-based composites and BCP20/80-based composites at day 1, 5, 7, and 12. Both composites were transferred to 24-well culture plates (Cellstar), washed with PBS, crushed in 300 µl Milli-Q water, and stored at -20°C prior to further use. ALP activity was measured in the cell lysate using 4-nitrophenyl phosphate disodium salt (Merck) as a substrate at pH 10.3, according to the method described by Lowry. The absorbance was read at 405 nm with a Synergy HT® spectrophotometer. ALP activity was expressed as μM per ng DNA.

**Nitric oxide**
NO production by BCP60/40-based composites and BCP20/80-based composites was measured as nitrite (NO$_2^-$) accumulation in conditioned medium at day 1, 5, 7, and 12 by using Griess reagent containing 2% sulphanilamide, 0.2% naphthylethelene-diamine-dihydrochloride, and 5% H$_3$PO$_4$. Serial dilutions of 0.1 M NaNO$_2$ in α-MEM were used as a standard curve. The absorbance was measured at 540 nm with a Synergy HT® spectrophotometer.

**Vascular endothelial growth factor production**
Human vascular endothelial growth factor (VEGF) production by BCP60/40-based composites and BCP20/80-based composites was measured in conditioned medium at day 1, 5, 7, and 12 by an enzyme-linked immunosorbent assay (DuoSet® ELISA, R&D Systems, Inc., Minneapolis, MN, USA). The absorbance was measured at 490 nm with a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). VEGF production was expressed in ng/ml.

**Fibrin degradation**
Fibrin degradation products were quantified using an enzyme-linked immunosorbent assay as described. Briefly, the antibody fibrin degradation products-14 (FDP-14; TNO, Quality of Life, Leiden, The Netherlands) recognizing different epitopes of fibrin degradation products was used as catching antibody. Fibrin degradation product concentrations in conditioned medium of BCP60/40-based composites and BCP20/80-based composites were investigated at day 1, 5, 7, and 12, and Biopool standard (Trinity Biotech, Wicklow, Ireland) was used as a reference. Finally, monoclonal antibody D-dimer-13 (DD-13; TNO) labeled with horseradish peroxidase was used as tagging antibody. The coloring reaction was performed using
3,3′,5,5′-tetramethybenzidine (Sigma-Aldrich, St. Louis, MO, USA) and stopped with 1 M H$_2$SO$_4$. The optical density was read at 450 nm with Synergy HT® spectrophotometer.

**Statistical analysis**

Data were obtained from quadruple cultures of two independent experiments for BCP60/40-based composites and BCP20/80-based composites. Data are presented as mean±SEM. To compare DNA content, ALP activity, NO production, VEGF production, and fibrin degradation for BCP60/40-based composites and BCP20/80-based composites at normoxic and hypoxic culture conditions, two-tailed unpaired t-test was performed. Differences were considered significant if p<0.05. Statistical analysis was performed using IBM® SPSS® Statistics version 21 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism® 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

**RESULTS**

**Hypoxia decreased total DNA content and ALP activity in BCP60/40-based composites and BCP20/80-based composites**

Total DNA content in BCP60/40-based composites and in BCP20/80-based composites was similar under normoxia and hypoxia at day 1 and 5, and decreased compared to normoxia at day 7 (BCP60/40: 1.3-fold; BCP20/80: 1.3-fold decrease) and day 12 (BCP60/40: 1.2-fold; BCP20/80: 1.2-fold decrease; Fig. 1A,B). Cell proliferation in BCP60/40-based composites increased by 1.2-fold under normoxia, and by 1.3-fold under hypoxia from day 1 to day 12. In BCP20/80-based composites, cell proliferation increased by 1.5-fold under normoxia, and by 1.3-fold under hypoxia from day 1 to day 12. ALP activity in BCP60/40-based composites and in BCP20/80-based composites under hypoxia was decreased compared to normoxia at day 5 (BCP60/40: 5.0-fold; BCP20/80: 4.7-fold decrease), day 7 (BCP60/40: 4.5-fold; BCP20/80: 6.0-fold decrease), and day 12 (BCP60/40: 7.1-fold; BCP20/80: 6.8-fold decrease; Fig. 1C,D).

**Hypoxia did not affect NO production by BCP60/40-based composites and BCP20/80-based composites**

NO production by BCP60/40-based composites was similar under normoxia and hypoxia at day 1, 5, 7, and 12 (Fig. 2A). Under normoxia, NO production by BCP60/40-based composites increased by 3.0-fold from day 1 to day 12, while under hypoxia, NO production was increased by 2.1-fold (Fig. 2A). There were also no differences in NO production by BCP20-based composites under normoxia and hypoxia at day 1, 5, 7, and 12 (Fig. 2B). NO production by BCP20/80-based composites increased by 5.5-fold under normoxia from day 1 to day 12, while NO production was increased by 3.3-fold under hypoxia (Fig. 2B).
Oxygen effect on hASCs on BCPs in fibrin gel

**Figure 1.** Total DNA content in (A) BCP60/40-based composites and (B) BCP20/80-based composites under normoxia and hypoxia at day 1, 5, 7, and 12. Hypoxia decreased total DNA content in (A) BCP60/40-based composites and (B) BCP20/80-based composites compared to normoxia at day 7 and day 12. ALP activity in (C) BCP60/40-based composites and (D) BCP20/80-based composites under normoxia and hypoxia at day 1, 5, 7, and 12. Hypoxia decreased ALP activity in (C) BCP60/40-based composites and (D) BCP20/80-based composites. Values are mean±SEM (n=4-8). *Significantly different from normoxia, p<0.05. BCP, biphasic calcium phosphate; ALP, alkaline phosphatase.

**Hypoxia enhanced VEGF production by BCP60/40-based composites and BCP20/80-based composites**

VEGF production by BCP20/80-based composites under hypoxia decreased by 2.1-fold, but not by BCP60/40-based composites, compared to normoxia at day 1 (Fig. 3). VEGF production by BCP60/40-based composites and BCP20/80-based composites under hypoxia was increased at day 5 (BCP60/40: 3.9-fold; BCP20/80: 3.8-fold), and day 7 (BCP60/40: 2.6-fold; BCP20/80: 2.5-fold), but reached similar levels at day (Fig. 3).
Figure 2. NO production by (A) BCP60/40-based composites and (B) BCP20/80-based composites under normoxia and hypoxia at day 1, 5, 7, and 12. Hypoxia did not affect NO production by (A) BCP60/40-based composites and (B) BCP20/80-based composites. Values are mean±SEM (n=6-8). BCP, biphasic calcium phosphate; NO, nitric oxide; cum, cumulative.

Figure 3. VEGF production by (A) BCP60/40-based composites and (B) BCP20/80-based composites under normoxia and hypoxia at day 1, 5, 7, and 12 days. Hypoxia enhanced VEGF production by (A) BCP60/40-based composites and (B) BCP20/80-based composites. Values are mean±SEM (n=8). *Significantly different from normoxia, p<0.05. BCP, biphasic calcium phosphate; VEGF, vascular endothelial growth factor; cum, cumulative.

Hypoxia did not affect fibrin degradation by BCP60/40-based composites and BCP20/80-based composites
Similar levels of fibrin degradation products in the medium of BCP60/40-based composites under hypoxia was observed compared to normoxia at day 1, 5, 7, and 12 (Fig. 4A).
Concentration of fibrin degradation products in the medium of BCP60/40-based composites increased by ~ 4000-fold under normoxia and hypoxia from day 1 to day 12 (Fig. 4A). There were also no differences in concentration of fibrin degradation products in the medium of BCP20/80-based composites under hypoxia compared to normoxia at day 1, 5, 7, and 12 (Fig. 4B). The level of fibrin degradation products in the medium of BCP20/80-based composites increased by ~ 13,000-fold under normoxia, and by ~ 4000-fold under hypoxia, from day 1 to day 12 (Fig. 4B).

**Figure 4.** Fibrin degradation of (A) BCP60/40-based composites and (B) BCP20/80-based composites under normoxia and hypoxia at day 1, 5, 7, and 12 days. Hypoxia did not affect fibrin degradation of (A) BCP60/40-based composites and (B) BCP20/80-based composites. Values are mean±SEM (n=7-8). BCP, biphasic calcium phosphate; cum, cumulative; fib, fibrin; deg, degradation.

**DISCUSSION**

We studied the effect of hypoxia on osteogenic and/or vasculogenic differentiation of hASC-seeded BCP60/40 or BCP20/80 scaffolds in fibrin gels as well as fibrin gel degradation. We found that (i) Hypoxia decreased total DNA content and ALP activity in BCP60/40-based composites and BCP20/80-based composites; (ii) Hypoxia did not affect NO production by BCP60/40-based composites and BCP20/80-based composites; (iii) Hypoxia increased VEGF production by BCP60/40-based composites and BCP20/80-based composites; (iv) Hypoxia did not affect fibrin degradation by BCP60/40-based composites and BCP20/80-based composites. Therefore, our results showed that hypoxia decreased osteogenic differentiation, and enhanced vasculogenic differentiation of BCP-based composites *in vitro*. This suggests that the hypoxic host microenvironment that cells might encounter after implantation *in vivo*, might enhance sprouting and tube formation by VEGF production, which increases survival of cells in BCP-based composites and stimulates bone formation.
Stem cells in vivo reside in a hypoxic microenvironment in which they must proliferate and differentiate. Hypoxia enhances hASC proliferation, and maintains stemness of hASCs. We found similar proliferation under normoxia and hypoxia in BCP60/40-based composites and BCP20/80-based composites, which indicates cell survival and maintenance of stemness of these cells in these composites. Hypoxia increases glucose consumption and lactate production of hASCs. This suggests that the hASCs in our BCP-composites under hypoxia compensate their metabolism more towards anaerobic glycolysis.

Hypoxia inducible factors (HIFs) are transcription factors, and their signaling pathway mediates the effects of hypoxia on cells. These transcription factors are essential for the maintenance of stemness of stem cells including hASCs and human embryonic stem cells. The stemness maintenance of hASCs under hypoxia allows stem cell differentiation towards specific lineages. Osteogenic differentiation potential of hASCs is reduced under hypoxia. This is in accordance with our results showing that hypoxia decreased ALP activity of hASCs in BCP60/40-based composites and BCP20/80-based composites, suggesting decreased osteogenic differentiation of hASCs in both BCP-based composites, and maintenance of stemness of hASCs under hypoxia.

HIF-1 is a heterodimeric transcription factor composed of two subunits: HIF-1α and HIF-1β. Low oxygen-induced HIF-1 increases VEGF expression. VEGF is a growth factor that plays an important role in e.g. vascular development, angiogenesis, and wound healing. Increased VEGF expression induces proliferation, sprouting, migration, and tube formation of endothelial cells. The upregulated VEGF production in BCP60/40-based composites and BCP20/80-based composites under hypoxia at earlier time points suggests that sprouting and tube formation are increased under hypoxia. We have attempted to determine the effect of hypoxia on formation of tubular structures in both composites after co-culture with primary human endothelial cells as described. This assay proved unsuitable for our composites, and future studies are needed to show possible differences in sprouting and tube formation in BCP-based composites under hypoxia compared to normoxia.

VEGF is expressed by hASCs as well as by osteoblasts. Hypoxia influences osteoblastic function, i.e. it up-regulates VEGF gene and protein expression, and increases ALP activity. The induction of VEGF expression by osteoblasts in response to hypoxia is modulated by an oxygen-sensing mechanism. Since osteogenesis and angiogenesis are tightly coupled processes, it might be expected that an upregulation of VEGF expression under hypoxia causes increased osteogenic differentiation of hASCs. However, we did not find such an increase in ALP activity as a result of hypoxia. This suggests that hypoxia influenced maintenance of cell stemness strongly, which prevents that a possible effect by VEGF on osteogenic stimulation can be expressed. We found similar VEGF production in BCP60/40-based composites and BCP20/80-based composites at the latest time point. This could be due to a hypoxic microenvironment for hASCs in BCP-composites under normoxia.
and hypoxia, which is in line with our previous study suggesting that VEGF expression increased in BCP20/80-based composites due to a hypoxic microenvironment for hASCs.\textsuperscript{17}

NO is an signaling molecule involved in many processes like inflammation, angiogenesis, and bone mass regulation. The molecule is synthesized by NO synthase (NOS) or by reduction of nitrite which can spontaneously occur under hypoxic and/or acidic conditions.\textsuperscript{35} Three different NOS isoforms exist: endothelial NO synthase (eNOS), neuronal NO synthase (nNOS), both calcium-dependent, and inducible form of NO synthase (iNOS) which is calcium-independent. All three isoforms are expressed in bone tissue.\textsuperscript{36} Low-oxygen-induced HIF-1 transcription factor increases iNOS and eNOS mRNA and protein expression.\textsuperscript{37-39} Therefore, an upregulation of NO production by BCP60/40-based composites and BCP20/80-based composites under hypoxia was expected, but similar NO production by BCP-based composites under normoxia and hypoxia was found. Since hypoxia did not affect NO production in BCP-based composites, this suggests similar spontaneous reduction of nitrite as well as induction of HIF-1 regulating iNOS and eNOS expression in both BCP-based composites under both conditions which is appealing for bone tissue engineering approaches.

Cells sense and respond to mechanical properties of their surroundings by locally pulling and pushing on the matrix, and at the same time actively change the stiffness and tension of their surroundings in a mechanical feed-back loop.\textsuperscript{40,41} Differences in matrix stiffness affect cell-mediated fibrin remodeling,\textsuperscript{42} which might have impact on this mechanical feed-back loop. The similar fibrin remodeling rates we found in BCP60/40-based composites and in BCP20/80-based composites under normoxia and hypoxia indicates no effect of hypoxia on matrix stiffness suggesting no impact on the mechanical feed-back loop between cells and matrix. This finding supports that our composites seem promising candidates for bone tissue engineering approaches like bone augmentation \textit{in vivo}.\textsuperscript{17}

In summary, hypoxia decreased DNA content and ALP activity in BCP60/40-based composites and BCP20/80-based composites, and increased VEGF production by both BCP-based composites. Therefore, we conclude that hypoxia decreased osteogenic differentiation potential, and enhanced vasculogenic differentiation potential of BCP60/40-based composites and BCP20/80-based composites \textit{in vitro}. This suggests that the hypoxic host microenvironment that cells will encounter after implantation \textit{in vivo} might enhance sprouting and tube formation by VEGF production which increases cell survival in BCP-based composites, and stimulates bone formation.
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