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RESEARCH ARTICLE

Optimization of culture conditions for osteogenically-induced mesenchymal stem cells in β-tricalcium phosphate ceramics with large interconnected channels

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Abstract

The aim of this study was to optimize culture conditions for human mesenchymal stem cells (hMSCs) in β-tricalcium phosphate ceramics with large interconnected channels. Fully interconnected macrochannels comprising pore diameters of 750 μm and 1400 μm were inserted into microporous β-tricalcium phosphate (β-TCP) scaffolds by milling. Human bone marrow-derived MSCs were seeded into the scaffolds and cultivated for up to 3 weeks in both static and perfusion culture in the presence of osteogenic supplements (dexamethasone, β-glycerophosphate, ascorbate). It was confirmed by scanning electron microscopic investigations and histological staining that the perfusion culture resulted in uniform distribution of cells inside the whole channel network, whereas the statically cultivated cells were primarily found at the surface of the ceramic samples. It was also determined that perfusion with standard medium containing 10% fetal calf serum (FCS) led to a strong increase (seven-fold) of cell numbers compared with static cultivation observed after 3 weeks. Perfusion with low-serum medium (2% FCS) resulted in moderate proliferation rates which were comparable to those achieved in static culture, although the specific alkaline phosphatase (ALP) activity increased by a factor of more than 3 compared to static cultivation. Gene expression analysis of the *ALP* gene also revealed higher levels of *ALP* mRNA in low-serum perfused samples compared to statically cultivated constructs. In contrast, gene expression of the late osteogenic marker bone sialoprotein II (*BSPII*) was decreased for perfused samples compared to statically cultivated samples.

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Keywords **β-tricalcium phosphate; channel-like pores; MSC; human bone marrow stromal cells; osteogenic differentiation; ALP; perfusion culture**

1. Introduction

Beta-tricalcium phosphate (β-TCP) ceramics are widely used in the field of bone regeneration because of their good biocompatibility and biodegradability. These ceramics are excellent bone grafts since the material can be completely replaced by new bone tissue, due to its high bioresorbability. The material itself has no osteoinductive properties, which means that β-TCP without osteogenic factors or osteogenic cells cannot give rise to the formation of new bone tissue (Heymann *et al.*, 2001; LeGeros *et al.*, 2002; Betz *et al.*, 2002; Liu *et al.*, 2007).

However, some authors have reported an enrichment of growth factors inside such ceramics, which is caused by capillary forces and leads to enhanced bone formation (Yamasaki and Sakai, 1992; Ripamonti, 1996; Yuan *et al.*, 1999; Habibovic *et al.*, 2006). The combination of porous β-TCP scaffolds with osteogenic cells can

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accelerate the bone formation process after implantation. Mesenchymal stem cells (MSCs), which can be isolated from bone marrow and other tissues, are an excellent source of osteoprogenitor cells for bone tissue-engineering approaches.

Pore distribution and pore size of biomaterials play a critical role in cell migration and proliferation, influencing both *in vitro* and *in vivo* bone formation (reviewed by Karageorgiou and Caplan, 2005). Various studies have been performed to evaluate the optimal pore size for osteogenesis; as a result, a minimal pore size of 100 μm has been claimed to enable cell ingrowth (Karageorgiou and Caplan, 2005; Klawitter and Hulbert, 1971), whereas pore sizes >200 μm are generally accepted to support new bone formation (Gauthier *et al*., 1998; Flautre *et al*., 2001; Galois *et al*., 2004).

However, scaffolds with very large pores or channels could be advantageous to prevent the retardation of fluid flow by growing cell layers and clusters. It was demonstrated that drilling holes in an allograft material improves subsequent bony ingrowth (Gendler *et al*., 1986). Furthermore, aligned macro-channels may provide a direction for bone ingrowth. Xu *et al*. (2007) cultivated rat MSCs in ceramic scaffolds with central aligned channels in the range 402–1988 μm and found rapid proliferation within 5 days, the highest cell area coverage of the channel walls being accomplished with a channel diameter of 789 μm. Von Doernberg *et al*. (2006) analysed the *in vivo* behaviour of β-TCP scaffolds with pore sizes of 150–1220 μm and found new bone formation in all types of scaffolds. In a theoretical study, Bohner and Baumgart (2004) evaluated a pore size of 800–1000 μm to be optimal for minimal resorption time of calcium phosphate ceramic scaffolds. Three-dimensional (3D) scaffolds with controlled architecture comprising fully interconnected pores with defined size and shape are considered to be beneficial for bone regeneration approaches. The main advantages of scaffolds with controlled architecture are adjusted mechanical properties and a reproducible degradation rate *in vivo* (Chu *et al*., 2002), as well as predicted fluid-flow configuration at medium perfusion (Wang *et al*., 2008).

The principle of tissue engineering involves the maturation of cell–scaffold constructs *in vitro* before implantation. This process can be hampered by reduced nutrient supply and mass transport, especially in scaffolds of clinically relevant size. To overcome these limitations, the application of medium perfusion for the *in vitro* cultivation of cell-seeded 3D scaffolds is beneficial. Perfusion culture is a well-established method which has been, and is still, widely investigated using various flow systems. In contrast to static cultivation, perfusion culture maintains an efficient concentration of nutrients and gases in the culture medium, allowing the survival and proliferation of cells even in the inner parts of large scaffolds. Furthermore, a positive impact of the shear stress originating from fluid flow in the perfusion culture on osteogenic differentiation has been observed (Datta *et al*., 2006; Zhao *et al*., 2007; Kreke *et al*., 2008; Li *et al*., 2009).

Static culture of MSCs always requires the addition of fetal or human serum. It was shown that serum-free media without further addition of growth factors are not capable of supporting the proliferation of MSCs (Berger *et al*., 2006) and that MSCs undergo apoptosis when subjected to hypoxia and serum deprivation (Zhu *et al*., 2006). However, as shown in the past, serum-free and low-serum conditions combined with perfusion culture were successful in the differentiation of epithelial cells (Minuth *et al*., 2001) and chondrocytes (Sittinger *et al*., 1997). Therefore, the investigation of low-serum conditions compared to standard conditions seems to be useful for the study of perfusion culture.

In the present study, cylindrical block geometries, consisting of pure phase β-TCP and controlled architecture, with an interconnecting macropore system of 750 μm and 1400 μm pore size combined with micropores in the range 0.1–50 μm (Cerasorb®; Peters and Reif, 2004; Peters *et al*., 2006; Tadic and Epple, 2004), were evaluated with regard to their suitability for *in vitro* expansion and osteogenic differentiation of human MSCs (hMSCs). Static culture was compared with perfusion culture under standard as well as low-serum conditions with respect to distribution, proliferation and osteogenic differentiation of hMSCs.

2. Materials and methods

2.1. Preparation of porous ceramics

Cylindrical samples (12 mm diameter, 6 mm height) from microporous phase pure β-TCP (Cerasorb) were prepared. Two different interconnected pore channel structures were inserted by milling. The preparation of the samples has been described in detail elsewhere (Peters *et al*., 2006). Briefly, pure phase β-TCP powder with a particle size of <63 μm was isostatically cold-pressed in order to make blank rods. By using a CAD/CAM device (VX), block geometries with ideal pore distribution were constructed and converted to the format of the milling device. The interconnected channel system, with 750 μm and 1400 μm channel widths, respectively, was milled using a HSPC milling machine (KERN, Germany) followed by an additional sintering process at ≥1000 °C. In the following, structured Cerasorb samples with 750 μm channel width are referred to as C750 and structured Cerasorb samples with 1400 μm channel width as C1400. The pattern of channel arrangement in milled Cerasorb samples is given in Figure 1A.

2.2. Cell culture

2.2.1. Cell seeding

hMSCs isolated from bone marrow aspirate from a healthy male donor (aged 33 years) were kindly

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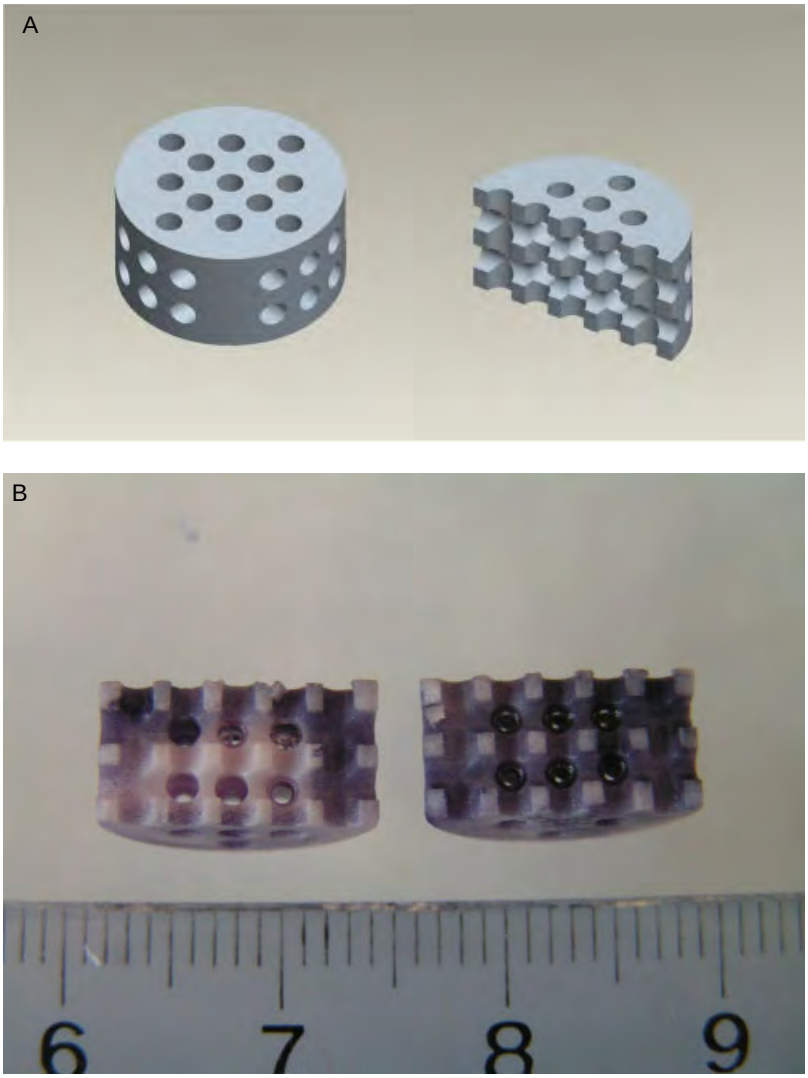

Figure 1. (A) Schematic image of channel arrangement in milled Cerasorb M samples with 1400 μm pore size (image: Th. Hänel, Technical University Chemnitz). (B) MTT staining of cell-seeded β-TCP samples C1400 after 21 days of static culture (left) and perfusion culture (right) under standard conditions. Scaffolds were broken into sections after performing MTT staining

provided by Professor M. Bornhäuser and his coworkers (Medical Clinic I, Dresden University Hospital Carl Gustav Carus). Expansion of the cells was performed in low-glucose Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS; Biochrom), 10 U/ml penicillin and 100 μg/ml streptomycin (Biochrom) at 37 °C in a humidified, 7% CO_2/93% air incubator. Cells from the fifth passage were used for seeding the ceramics. All procedures were approved by the Ethical Commission of the Medical Faculty of the Technical University, Dresden.

Prior to cell seeding, the ceramics were pre-incubated with cell culture medium for 24 h. For cell seeding, equilibration medium was removed. Samples were placed in 48-well polystyrene culture dishes. 100 μl cell culture medium containing 2×10^5 cells was applied to each cylindrical sample. After 1 h of initial adhesion, 1 ml cell culture medium was added to each sample. After a further 24 h of cultivation, cell-seeded samples were transferred to fresh culture dishes or into perfusion chambers (see below).

2.2.2. Static cultivation of cell-seeded β-TCP scaffolds

For static experiments, cell-seeded ceramics were cultivated in 24-well dishes with 1 ml cell culture medium/sample. The medium was changed twice weekly. Static culture was performed in DMEM containing 10% FCS and antibiotics in the presence of osteogenic supplements (OS; 10^{-7} M dexamethasone, 3.5 mM β-glycerophosphate and 0.05 mM ascorbic acid 2-phosphate; Sigma, Taufkirchen, Germany).

2.2.3. Perfusion cultivation of cell-seeded β-TCP scaffolds

For perfusion experiments, cell-seeded ceramics were cultivated in a commercially available perfusion system

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(Minucells, Germany). Up to eight samples were set into each perfusion chamber, having an internal diameter of 13 mm. A continuous medium flow provided by an eight-channel peristaltic pump, set at 1.5 ml/h, allowing the perfusion of several chambers simultaneously. After passing the perfusion chamber, the medium was collected in a waste bottle, fresh medium being refilled every 2–3 days. Perfusion culture was performed in the presence of OSs (10^{-7} M dexamethasone, 3.5 mM β-glycerophosphate and 0.05 mM ascorbic acid 2-phosphate; Sigma) with two different media: DMEM containing 10% FCS, antibiotics and OS (standard conditions); and DMEM containing 2% FCS, antibiotics and OS (low-serum conditions).

2.3. Biochemical analysis of alkaline phosphatase (ALP) activity and DNA content

Samples for biochemical measurements ($n = 3$) were taken at days 1, 14 and 21 of cultivation, washed with phosphate-buffered saline (PBS) and frozen until further analysis. Frozen cell-seeded ceramics were thawed for 20 min on ice, followed by lysis with 1% Triton X-100 in PBS for 50 min on ice. During cell lysis, the samples were sonicated for 10 min in an ultrasonic bath (Bandelin, Sonorex TK 52, Berlin, Germany).

One aliquot of the cell lysates was added to an ALP reaction buffer containing 1 mg/ml *p*-nitrophenyl phosphate (Sigma), 0.1 M diethanolamine, 1% Triton X-100, pH 9.8, and 1 mM $MgCl_2$. This mixture was incubated at 37 °C for 30 min. Finally, 1 M NaOH was added to stop the enzymatic reaction. After centrifugation at 16 000 × *g* for 10 min, the supernatant was transferred to a microtitre plate and the absorbance was read at 405 nm with a multifunction microplate reader (Spectra Fluor Plus, Tecan, Crailsheim, Germany). A calibration line was constructed from different dilutions of a 1 mM *p*-nitrophenol stock solution.

Another aliquot of the cell lysates was mixed with Picogreen ds DNA quantitation reagent (Molecular Probes, Eugene, OR, USA) diluted 1 : 800 in TE buffer (= 10 mM TRIS and 1 mM EDTA) and incubated for 5 min in the dark. The intensity of fluorescence was measured with a multifunction microplate reader (Spectra Fluor Plus) at excitation and emission wavelengths of 485/535 nm. Relative fluorescence units were correlated with the cell number using a calibration line.

2.4. Scanning electron microscopy (SEM)

Cell-seeded ceramics were washed twice with PBS, fixed for 30 min with 3.7% formaldehyde in PBS, washed with distilled water and dehydrated using a graded series of ethanol–distilled water solutions. Critical point drying was performed with a CPD 030 apparatus (BAL-TEC, Liechtenstein). The samples were broken into sections, coated with gold and imaged using a Philips XL 30/ESEM, with a field emission gun operating in SEM mode. The microscope was driven with an acceleration voltage of 5 kV and a working distance of 14 mm detecting secondary electrons.

2.5. MTT staining

After cultivation of the cell-seeded ceramics for 21 days, the samples were transferred from the perfusion chamber to 24-well cell culture dishes and supplemented with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma), followed by further incubation at 37 °C for 4 h. The formation of dark purple formazan dye converted from MTT by mitochondrial dehydrogenases of living cells was documented photographically.

2.6. Reverse transcriptase PCR

RNA was isolated on days 14 and 21 of cultivation ($n = 3$), using the peqGOLD MicroSpin Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. cDNA was transcribed from 250 ng total RNA in a 20 µl reaction mixture containing 200 U Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA), 0.5 mM dNTPs (Invitrogen), 12.5 ng/µl random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U RNase inhibitor RNase OUT (Invitrogen). For PCR experiments, 1 µl cDNA was amplified in a 20 µl reaction mixture containing 1.5 U HotTaq-Polymerase (Peqlab), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and specific primer pairs (1 µM of each primer) to detect transcripts of *ALP, osteopontin, BSPII, osteonectin* and the housekeeping gene *GAPDH*, respectively, for each sample in a thermocycler (Peqlab). The sequences and annealing temperatures of the primers

Table 1. Primers for RT–PCR

Gene	Primer sequences	$T_{annealing}$ (°C)	Product size (bp)
ALP	F: 5′-ACCATTCCCACGTCTTC ACATTTG-3′ R: 5′-ATTCTCTCGTTCACCGC CCAC-3′	55	162
Osteopontin	F: 5′-GTCTCAGGCCAGTTGCA GCC-3′ R: 5′-GCCATGTGGCCACAGC ATCTG-3′	59	187
BSPII	F: 5′-AATGAAAACGAAGAA AGCGAAG-3′ R: 5′-ATCATAGCCATCGTAGC CTTGT-3′	55	450
Osteonectin	F: 5′-ATCTTCCCTGTACACTG GCAGTTC-3′ R: 5′-CCACTCATCCAGGGCGA TGTAC-3′	57	177
GAPDH	F: 5′-GGTGAAGGTCGGAGTCA ACGG-3′ R: 5′-GGTCATGAGTCCTTCC ACGAT-3′	55	520

F, forward; R, reverse.

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(MWG Biotech) for each gene, as well as the sizes of the PCR products, are summarized in Table 1. The same single-stranded cDNA was used to analyse the expression of all genes described. The resulting PCR products were visualized using the FlashGel™ Dock system (Cambrex Bio Science, Rockland, USA). Expression of the osteogenic markers ALP, osteopontin, BSPII and osteonectin was normalized to the expression of the housekeeping gene *GAPDH* by image analysis, using the Bio Imaging System Gene Genius with the acquisition software Gene Snap and the analysis software Gene Tools (SynGene, Cambridge, UK).

2.7. Statistics

Data on biochemical measurements are represented by the means of three individual samples. Error bars represent standard deviation (SD). Statistical comparisons were made by Student's *t*-test. A difference was considered significant at $p < 0.05$.

3. Results

3.1. Perfusion culture under standard conditions led to a more uniform cell distribution in the inner parts of the scaffolds and promoted cell proliferation

3.1.1. Distribution of cells within the interconnected channel network

The intracellular conversion of MTT to insoluble formazan was used to visualize the distribution of living cells within the porous ceramics. This method is appropriate to evaluate cell allocation as well as viability within a whole ceramic section at a glance and had already been applied by us and others for this purpose (Mauney *et al*., 2004; Du *et al*., 2008; Lode *et al*., 2008; Gelinsky *et al*., 2008).

After static cultivation of cell-seeded ceramics, the majority of living cells were detected near the surface of the scaffolds. In the case of perfusion culture, uniform staining was detected through the whole scaffold (Figure 1B).

SEM investigations of scaffold sections revealed penetration of cells into the channels in both static and perfusion culture. However, in the case of perfusion culture a thicker cell layer was observed in channels distant from the scaffold surface (Figure 2).

3.1.2. Cell proliferation

After 3 weeks of static cultivation, the number of osteogenically-induced hMSCs on C750 and C1400 was increased by a factor of 2.2 and 2.6, respectively, compared to day 1. A highly significant increase of cell numbers after 3 weeks of cultivation was found when the cell-seeded ceramics were cultivated in the perfusion system. In this case, cell numbers rose by a factor of almost 16 on both C750 and C1400 (Figure 3).

3.1.3. Alkaline phosphatase (ALP) activity

ALP activity related to cell number (specific ALP activity) in both static and perfusion culture of cell-seeded ceramics increased from day 1 to day 21 of cultivation. Specific ALP activity was increased by a factor of 3 after 21 days of cultivation in both static and perfusion-cultivated samples (Figure 4). Thus, perfusion cultivation under standard conditions did not affect specific ALP activity in the studied scaffolds.

3.2. Perfusion culture under low-serum conditions promoted osteogenic differentiation in terms of ALP activity and expression, but not cell proliferation

3.2.1. Cell distribution

Perfusion culture under low-serum conditions also led to a uniform distribution of cells in the inner parts of the macroporous scaffolds, which was demonstrated by SEM investigations of the scaffold sections (Figure 5).

3.2.2. Cell proliferation

After 3 weeks of cultivation, the number of osteogenically-induced hMSCs on C750 and C1400 was increased by a factor of 2.2–3, respectively, compared to day 1. There were no significant differences between static cultivated samples and samples cultivated under perfusion culture with low-serum medium, indicating that low-serum perfusion will not enhance cell proliferation (Figure 6).

3.2.3. ALP activity

Specific ALP activity in both static and perfusion culture of cell-seeded ceramics rose from day 1 to day 21 of cultivation. While the ALP activity for static cultivation under standard conditions increased by a factor of >3, ALP activity for perfused samples under low-serum conditions increased by a factor of >10 (Figure 7). Thus, perfusion cultivation under low-serum conditions indicates an increase of specific ALP activity of osteogenically-induced hMSCs on the studied porous bioceramic scaffolds.

3.2.4. Gene expression of ALP, osteopontin, BSPII and osteonectin

Gene expression analysis was performed for static cultivated cell-seeded samples under standard conditions as well as for samples cultivated under perfusion culture with low serum-containing medium (Figure 8). After both 14 and 21 days of cultivation the gene expression of *ALP* was clearly higher for samples cultivated under medium perfusion. This is in accordance with ALP

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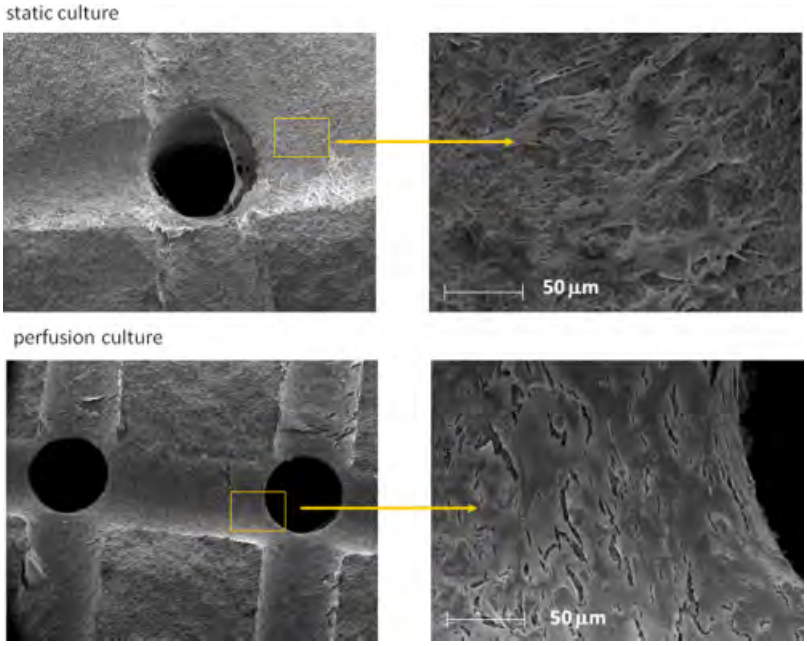

Figure 2. SEM images showing cross-sections of cell-seeded β-TCP samples C750 after 21 days of static culture as well as perfusion culture under standard conditions

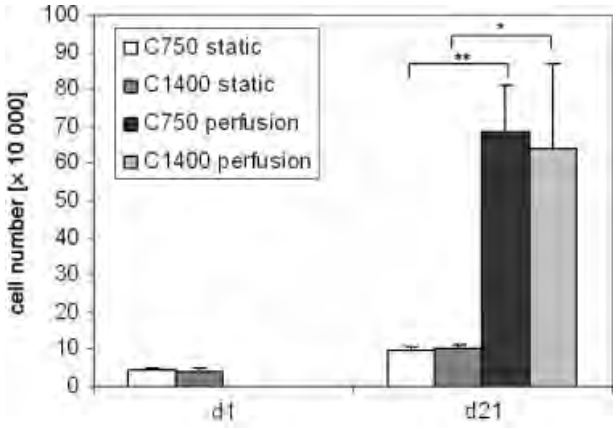

Figure 3. Proliferation of hMSCs cultivated with osteogenic supplements on C750 and C1400 samples in static and perfusion culture with standard medium ($n = 3 \pm$ standard deviation of the mean). Cell number was calculated from DNA content using a calibration line of known cell numbers, significant differences between static and perfusion culture ($^{*}p < 0.05$, $^{**}p < 0.01$)

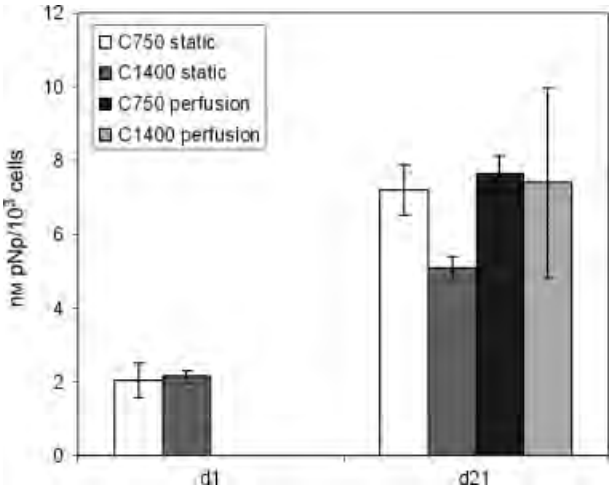

Figure 4. Specific ALP activity of hMSCs cultivated with osteogenic supplements on C750 and C1400 samples in static and perfusion culture with standard medium ($n = 3 \pm$ standard deviation of the mean). ALP activity was related to cell number, calculated from DNA content

activity measurements. Similar findings were achieved for *osteopontin* gene expression, predominately when cell-seeded samples of C750 were used. In the case of C1400 samples, no clear difference between *osteopontin* gene expression of static and perfused samples was detected. The gene expression of *osteonectin* was similar for static and perfused samples in both C750 and C1400 samples. In contrast, a clear increase of gene expression of the late osteogenic marker *BSPII* was detected only in statically cultivated C750 samples after 14 and 21 days of cultivation. For perfused C750 as well as for statically cultivated C1400 samples, no *BSPII* expression was observed after 14 days of cultivation and after 21 days a much lower *BSPII* expression was observed compared to static cultivation. For perfused C1400 samples, almost no *BSPII* expression was found.

4. Discussion

In the present study, hMSCs were successfully expanded in β-TCP samples with large interconnected channels. We found a better distribution of cells inside the pore systems of the scaffolds under perfusion. Uniform distribution of cells inside 3D scaffolds was frequently observed during perfusion culture of various cell-seeded materials (Goldstein *et al.*, 2001; Leukers *et al.*, 2005;

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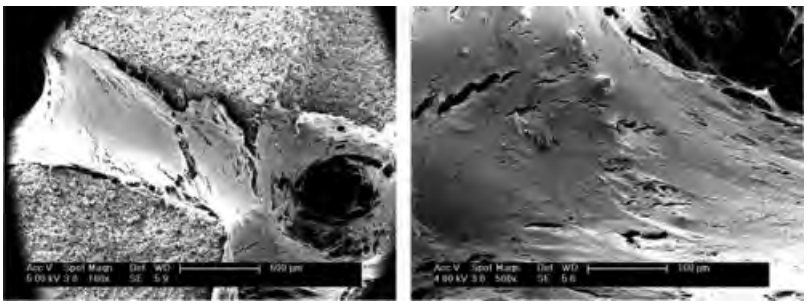

Figure 5. SEM images, showing cross-sections of cell-seeded β-TCP samples C750 after 21 days of perfusion culture under low-serum conditions

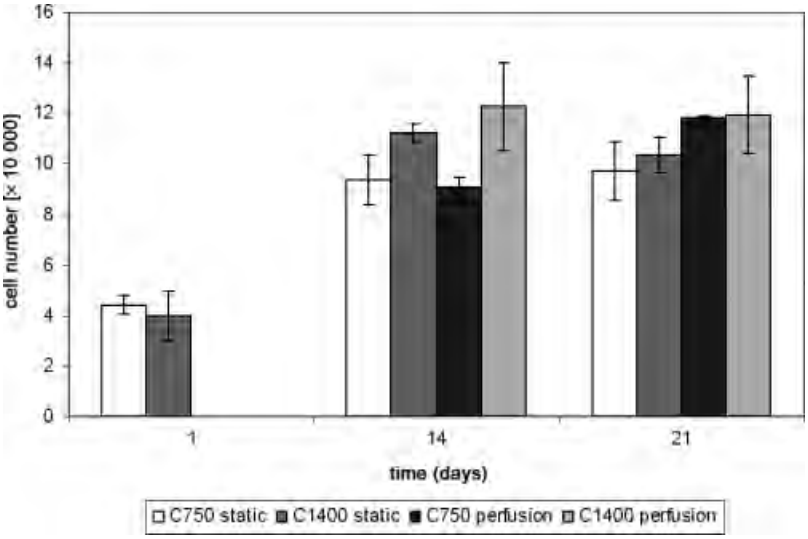

Figure 6. Proliferation of hMSCs cultivated with osteogenic supplements on C750 and C1400 samples in static culture under standard conditions and perfusion culture under low-serum conditions ($n = 3 \pm$ standard deviation of the mean). Cell number was calculated from DNA content using a calibration line of known cell numbers

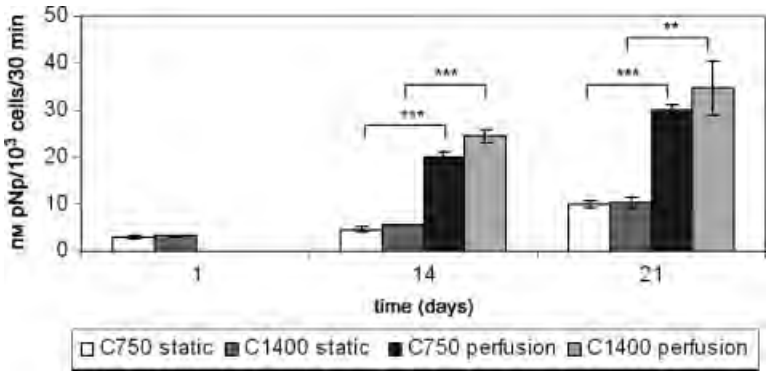

Figure 7. Specific ALP activity of hMSCs cultivated with osteogenic supplements on C750 and C1400 samples in static culture under standard conditions compared to perfusion culture with low-serum medium ($n = 3 \pm$ standard deviation of the mean). ALP activity was related to cell number, calculated from DNA content. Significant differences between static and perfusion culture ($^{**}p < 0.01$, $^{***}p < 0.001$)

Detsch *et al*., 2008; Meretoja *et al*., 2008; and others) and can be associated with the improved supply of nutrients and gases. These improved conditions can also result in an increased cell proliferation, which has been reported by further studies (Cartmell *et al*., 2003; van den Dolder *et al*., 2003; Hosseinkhani *et al*., 2005; Fassina *et al*., 2005). In our study, perfusion with standard cell culture medium containing 10% FCS led to a surge of cell numbers within 21 days of cultivation. Similar studies involving perfusion culture of large macroporous β-TCP scaffolds were performed by other groups. Xie *et al*. (2006) cultivated sheep MSCs in cylindrical macroporous β-TCP scaffolds containing an additional central channel. Perfusion culture resulted

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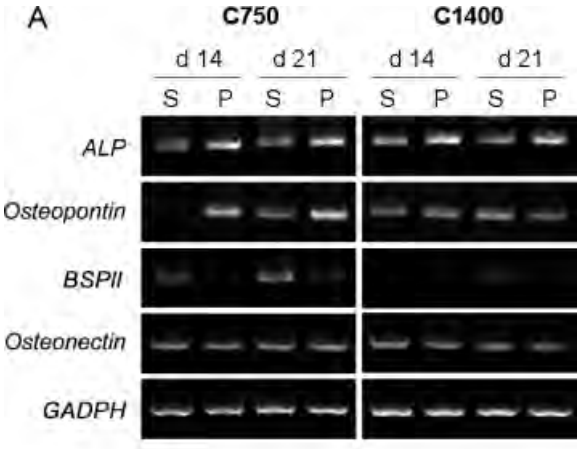

B relative expression normalized to GADPH

	C 750				C 1400			
	d 14		d 21		d 14		d 21	
	S	P	S	P	S	P	S	P
ALP	0.74	1.00	0.63	0.92	0.62	0.80	0.62	0.77
Osteopontin	0.10	0.94	0.65	1.00	0.55	0.62	0.73	0.44
BSPII	0.76	0.11	1.00	0.55	0.16	0.05	0.59	0.11
Osteonectin	0.99	0.97	0.94	1.00	0.88	0.77	0.88	0.75

Figure 8. Gene expression of *ALP, osteopontin, BSPII, osteonectin* and *GAPDH* for osteogenic-induced hMSCs seeded on C750 and C1400 scaffolds after 14 and 21 days of static cultivation under standard conditions, compared with perfusion culture under low-serum conditions. Analysis was performed for hMSCs from two donors with similar results (data for the second donor not shown). (A) Images of the agarose gels. (B) Expression of the osteogenic markers was normalized to the expression of the housekeeping gene *GAPDH*. For better comparison, the highest value was set at 1.0. S, static; P, perfusion

in higher cell viability and uniform distribution of cells throughout the whole scaffold. Li *et al*. (2008) analysed perfusion cultivation of rat calvaria osteoblasts in β-TCP scaffolds with 300–500 µm pore size and controlled pore distribution. They reported greater scaffold cellularity and higher levels of ALP activity in perfusion culture compared to static cultivation as well as homogeneous distribution of the cells inside the channel network of perfused samples. Another study involved perfusion culture of large β-TCP cylinders with 500 µm macropores arranged in controlled intervals seeded with human fetal osteoblasts (Wang *et al*., 2009). Compared to static culture, an improved cell proliferation and osteogenic differentiation was stated and a homogeneous cell layer was observed from the surface to the central parts of the scaffolds (Wang *et al*., 2009).

In contrast to the above-mentioned studies involving large macroporous β-TCP scaffolds (Li *et al*., 2008; Wang *et al*., 2009), our study evaluates an excessive increase of cell number obtained during perfusion with standard medium, which was not accompanied by an increased osteogenic differentiation of the cells. Under standard conditions, specific ALP activity of cells in perfused β-TCP samples was no higher than that of statically cultivated samples. It is well known that the proliferation and differentiation of cells are indirectly proportional, and the enhanced proliferation may be an explanation for the attenuated specific ALP activity during perfusion with 10% FCS. Another reason for the lack of ALP increase under standard perfusion conditions could be the very high channel width in the investigated scaffolds. Mygind *et al*. (2007) investigated the dynamic cultivation of hMSCs in scaffolds of 200 µm compared to 500 µm pore size. They detected a faster rate of osteogenic differentiation on scaffolds with smaller pores, whereas in the scaffolds with 500 µm pore size proliferation was favoured. Cell differentiation can be initiated by the withdrawal of growth factors or fetal serum (Minuth *et al*., 2000). To improve the osteogenic differentiation of hMSCs on perfused β-TCP samples, we initiated further experiments involving low-serum conditions (2% FCS). Upon reduction of FCS content, proliferation rates of osteogenically induced cells on β-TCP samples dropped but were nevertheless in the same range as proliferation rates of cells which were statically expanded using 10% FCS. Serum reduction combined with perfusion culture in the present study resulted in a significant increase of specific ALP activity compared to static cultivation using standard medium. Other groups also demonstrated an enhanced cell differentiation for epithelial cells and chondrocytes when subjected to perfusion culture under serum-free and low-serum conditions (Minuth *et al*., 2001; Sittinger *et al*., 1997). Gene expression analysis of osteogenic markers *ALP, osteopontin, BSPII* and *osteonectin* was carried out to further evaluate the influence of perfusion culture under low-serum conditions on the osteogenic differentiation of hMSCs in macroporous β-TCP scaffolds. In accordance with the ALP activity measurements, *ALP* gene expression was noticeably increased in perfusion culture with low-serum medium. We furthermore found that perfusion promoted *osteopontin* gene expression when scaffolds with 750 µm channel size were used. Augmentation of *osteopontin* expression of MSCs by perfusion culture has also been reported by many other authors (Sharp *et al*., 2009; Froehlich *et al*., 2010; Bjerre *et al*., 2008). Gene expression of *osteonectin* was not elevated by perfusion culture in our study, which is consistent with the results of Janssen *et al*. (2010), who found no significant differences between static and perfusion culture of hMSCs in macroporous biphasic calcium phosphate scaffolds. In contrast to these findings and our study, Zhao *et al*. (2009) reported an increase of osteonectin gene expression in 3D poly(ethylene terephthalate) scaffolds, even at low–medium velocities. Contrary to the other markers, expression of the late osteogenic marker BSPII was decreased under the selected perfusion conditions in our study. Mygind and co-workers also stated a decreased gene expression of BSPII for hMSCs in dynamic cultivated porous scaffolds compared to static cultivation (Mygind *et al*., 2007). In contrast, Bjerre *et al*. (2008) did not find an attenuation of BSPII expression upon dynamic cultivation. They cultivated hMSCs in silicate-substituted TCP scaffolds, using vitamin D3 as the only osteogenic stimulus, and reported a significant increase

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of osteogenic gene expression, including *ALP* and *BSPII*, when comparing perfusion and static culture (Bjerre *et al*., 2008). Possibly, the effect of medium perfusion is pronounced when the medium is supplemented not with dexamethasone but with vitamin D3. Furthermore, the applied flow rate of the respective study was a factor of 6 higher compared to the flow rate applied in our study, which may have caused much higher shear stress. Shear stress, applied by steady and pulsatile flow, was found to increase the osteogenic gene expression (including BSPII) of BMSCs (Kreke *et al*., 2005; Sharp *et al*., 2009).

In vitro expansion and osteogenic differentiation of MSCs in 3D scaffolds appears to be a promising approach for the delivery of a mature osteoblastic cell population, as it allows rapid bone formation in and around the implant material. Keeping in mind the different response of hMSCs to serum concentration under perfusion culture in our experiment, a two-step approach could be imaginable: first, MSCs are extensively expanded in the 3D scaffold; second, the cells are differentiated under low-serum conditions.

In the present study we also evaluated the influence of channel diameter on cell in-growth, proliferation and osteogenic differentiation. The channel diameter of the ceramic samples should influence the fluid flow and therefore the shear stress cells are exposed to perfusion culture. In our experiments we did not detect differences in proliferation when hMSCs were cultivated on scaffolds with 750 µm compared to 1400 µm channel diameter, either in static or in perfusion culture. These results suggest that ceramics with both channel sizes are suitable for the colonization of hMSCs. Our results on gene expression of osteogenic markers on scaffolds with different pore sizes suggest that the effect of perfusion culture on osteogenic differentiation is more advantageous in β-TCP scaffolds with 750 µm channel width compared to those with 1400 µm channel width. This effect may be explained by the higher shear forces in 750 µm scaffolds. Moreover, the above-cited study of Mygind and co-workers (2007) also stated a higher expression of osteogenic markers for hMSCs cultivated in scaffolds with 200 µm pores compared to 500 µm pores, suggesting that high pore sizes may not always be beneficial for the promotion of osteogenic differentiation. Possibly the pores applied to our experiments were above a certain threshold to make a difference in osteogenic differentiation. Conversely, it is conceivable that revised experimental parameters (perfusion flow rate, seeded cell number/scaffold) could reveal differences in proliferation and osteogenic differentiation between scaffolds of the two examined channel diameters.

5. Conclusions

β-TCP ceramics with large interconnecting macrochannels, several hundred micrometers in diameter, can be applied for the expansion and osteogenic differentiation of hMSCs. Perfusion culture of cell-seeded scaffolds under standard conditions promotes uniform cell distribution in the inner parts of the scaffolds and accelerates cell proliferation. The early stages of osteogenic differentiation of hMSCs seeded onto β-TCP ceramics with macrochannels can be improved by perfusion culture under low-serum conditions.

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References

Berger MG, Veyrat-Masson R, Rapatel C, *et al*. 2006; Cell culture medium composition and translational adult bone marrow-derived stem cell research. *Stem Cells* **24**: 2888–2890.

Betz RR. 2002; Limitations of autograft and allograft: new synthetic solutions. *Orthopedics* **25**(5 suppl): s561–570.

Bjerre L, Buenger CE, Moustapha Kassem M, *et al*. 2009; Flow perfusion culture of human mesenchymal stem cells on silicate-substituted tricalcium phosphate scaffolds. *Biomaterials* **29**: 2616–2627.

Bohner M, Baumgart F. 2004; Theoretical model to determine the effects of geometrical factors on the resorption of calcium phosphate bone substitutes. *Biomaterials* **25**: 3569–3582.

Cartmell SH, Porter BD, García AJ, *et al*. 2003; Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs *in vitro*. *Tissue Eng* **9**: 1197–1203.

Chu TM, Orton DG, Hollister SJ, *et al*. 2002; Mechanical and *in vivo* performance of hydroxyapatite implants with controlled architectures. *Biomaterials* **23**: 1283–1293.

Datta N, Pham QP, Sharma U, *et al*. 2006; *In vitro* generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. *Proc Natl Acad Sci USA* **103**: 2488–2493.

Detsch R, Uhl F, Deisinger U, *et al*. 2008; 3D cultivation of bone marrow stromal cells on hydroxyapatite scaffolds fabricated by dispense-plotting and negative mould technique. *J Mater Sci Mater Med* **19**: 1491–1496.

Du D, Furukawa K, Ushida T. 2008; Oscillatory perfusion seeding and culturing of osteoblast-like cells on porous beta-tricalcium phosphate scaffolds. *J Biomed Mater Res A* **86**: 796–803.

Fassina L, Visai L, Asti L, *et al*. 2005; Calcified matrix production by SAOS-2 cells inside a polyurethane porous scaffold, using a perfusion bioreactor. *Tissue Eng* **11**: 685–700.

Flautre B, Descamps M, Delecourt C, *et al*. 2001; Porous HA ceramic for bone replacement: role of the pores and interconnections – experimental study in the rabbit. *J Mater Sci Mater Med.* **12**: 679–682.

Fröhlich M, Grayson W, Marolt D, *et al*. 2010; Bone grafts engineered from human adipose-derived stem cells in perfusion bioreactor culture. *Tissue Eng Part A* **16**: 179–189.

Galois L, Mainard D. 2004; Bone ingrowth into two porous ceramics with different pore sizes: an experimental study. *Acta Orthop Belg* **70**: 598–603.

Gauthier O, Bouler JM, Aguado E, *et al*. 1998; Macroporous biphasic calcium phosphate ceramics: influence of macropore diameter and macroporosity percentage on bone ingrowth. *Biomaterials* **19**: 133–139.

Gelinsky M, Welzel PB, Simon P, *et al*. 2008; Porous three-dimensional scaffolds made of mineralised collagen: preparation and properties of a biomimetic nanocomposite material for tissue engineering of bone. *Chem Eng J* **137**: 84–96.

Gendler E. 1986; Perforated demineralized bone matrix: a new form of osteoinductive biomaterials. *J Biomed Mater Res* **20**: 687–697.

Goldstein AS, Juarez TM, Helmke CD, *et al*. 2001; Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. *Biomaterials* **22**: 1279–1288.

Gomes ME, Sikavitsas VI, Behravesh E, *et al*. 2003; Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. *J Biomed Mater Res A* **67**: 87–95.

Habibovic P, Sees TM, van den Doel MA, *et al*. 2006; Osteoinduction by biomaterials – physicochemical and structural influences. *J Biomed Mater Res A* **77**: 747–762.

Heymann D, Delécrin J, Deschamps C, *et al*. 2001; *In vitro* assessment of combining osteogenic cells with macroporous calcium-phosphate ceramics. *Rev Chir Orthop Reparatrice Appar Mot* **87**: 8–17.

Hosseinkhani H, Inatsugu Y, Hiraoka Y, *et al*. 2005; Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic acid) fiber. *Tissue Eng* **11**: 1476–1488.

Janssen FW, van Dijkhuizen-Radersma R, Van Oorschot A, *et al*. 2010; Human tissue-engineered bone produced in clinically relevant amounts using a semi-automated perfusion bioreactor system: a preliminary study. *J Tissue Eng Regen Med* **4**: 12–24.

Karageorgiou V, Kaplan D. 2005; Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* **26**: 5474–5491.

Klawitter JJ, Hulbert SF. 1971; Application of porous ceramics for the attachment of load-bearing orthopedic applications. *J Biomed Mater Res Symp* **2**: 161.

Kreke MR, Huckle WR, Goldstein AS. 2005; Fluid flow stimulates expression of osteopontin and bone sialoprotein by bone marrow stromal cells in a temporally dependent manner. *Bone* **36**: 1047–1055.

Kreke MR, Sharp LA, Lee YW, *et al*. 2008; Effect of intermittent shear stress on mechanotransductive signaling and osteoblastic differentiation of bone marrow stromal cells. *Tissue Eng A* **14**: 529–537.

LeGeros RZ. 2002; Properties of osteoconductive biomaterials: calcium phosphates. *Clin Orthop Relat Res* **395**: 81–98.

Leukers B, Gülkan H, Irsen SH, *et al*. 2005; Hydroxyapatite scaffolds for bone tissue engineering made by 3D printing. *J Mater Sci Mater Med* **16**: 1121–1124.

Li X, Li D, Wang L, *et al*. 2008; Osteoblast cell response to β-tricalcium phosphate scaffolds with controlled architecture in flow perfusion culture system. *J Mater Sci Mater Med* **19**: 2691–2697.

Li D, Tang T, Lu J, *et al*. 2009; Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. *Tissue Eng Part A* **15**: 2773–2783.

Liu G, Zhao L, Cui L, *et al*. 2007; Tissue-engineered bone formation using human bone marrow stromal cells and novel β-tricalcium phosphate. *Biomed Mater* **2**: 78–86.

Lode A, Bernhardt A, Gelinsky M. 2008; Cultivation of human bone marrow stromal cells on three-dimensional scaffolds of mineralized collagen: influence of seeding density on colonization, proliferation and osteogenic differentiation. *J Tissue Eng Regen Med* **2**: 400–407.

Mauney JR, Blumberg J, Pirun M, *et al*. 2004; Osteogenic differentiation of human bone marrow stromal cells on partially demineralized bone scaffolds *in vitro*. *Tissue Eng* **10**: 81–92.

Meretoja VV, Malin M, Seppälä JV, *et al*. 2008; Osteoblast response to continuous phase macroporous scaffolds under static and dynamic culture conditions. *J Biomed Mater Res A* **89**: 317–325.

Minuth WW, Schumacher K, Strehl R, *et al*. 2000; Physiological and cell biological aspects of perfusion culture technique employed to generate differentiated tissues for long term biomaterial testing and tissue engineering. *J Biomater Sci Polym Ed* **11**: 495–522.

Minuth WW, Strehl R, Schumacher K, *et al*. 2001; Long term culture of epithelia in a continuous fluid gradient for biomaterial testing and tissue engineering. *J Biomater Sci Polym Ed* **12**: 353–365.

Mygind T, Stiehler M, Baatrup A, *et al*. 2007; Mesenchymal stem cell ingrowth and differentiation on coralline hydroxyapatite scaffolds. *Biomaterials* **28**: 1036–1047.

Peters F, Reif D. 2004; Functional materials for bone regeneration from β-tricalcium phosphate. *Mat Wiss Werkstofftech* **35**: 203–207.

Peters F, Groisman D, Davids R, *et al*. 2006; Comparative study of patient individual implants from β-tricalcium phosphate made by different techniques based on CT data. *Mat Wiss Werkstofftech* **37**: 457–461.

Ripamonti U. 1996; Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. *Biomaterials* **17**: 31–35.

Sharp LA, Lee YW, Goldstein AS. 2009; Effect of low-frequency pulsatile flow on expression of osteoblastic genes by bone marrow stromal cells. *Ann Biomed Eng* **37**: 445–453.

Sittinger M, Schulz O, Minuth WW, *et al*. 1997; Artificial tissues in perfusion culture. *Int J Artif Organs* **20**: 57–62.

van den Dolder J, Bancroft GN, Sikavitsas VI, *et al*. 2003; Flow perfusion culture of marrow stromal osteoblasts in titanium fiber mesh. *J Biomed Mater Res A* **64**: 235–241.

von Doernberg MC, von Rechenberg B, Bohner M, *et al*. 2006; *In vivo* behavior of calcium phosphate scaffolds with four different pore sizes. *Biomaterials* **27**: 5186–5198.

Wang L, Hu YY, Wang Z, *et al*. 2009; Flow perfusion culture of human fetal bone cells in large β-tricalcium phosphate scaffold with controlled architecture. *J Biomed Mater Res A* **91**: 102–113.

Xie Y, Hardouin P, Zhu Z, *et al*. 2006; Three-dimensional flow perfusion culture system for stem cell proliferation inside the critical-size β-tricalcium phosphate scaffold. *Tissue Eng* **12**: 3535–3543.

Xu S, Li D, Wang C, *et al*. 2007; Cell proliferation in CPC scaffold with a central channel. *Biomed Mater Eng* **17**: 1–8.

Yamasaki H, Sakai H. 1992; Osteogenic response to porous hydroxyapatite ceramics under the skin of dogs. *Biomaterials* **13**: 308–312.

Yuan H, Kurashina K, de Bruijn JD, *et al*. 1999; A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics. *Biomaterials* **20**: 1799–1806.

Zhao F, Chella R, Ma T. 2007; Effects of shear stress on 3D human mesenchymal stem cell construct development in a perfusion bioreactor system: experiments and hydrodynamic modelling. *Biotechnol Bioeng* **96**: 584–595.

Zhao F, Grayson WL, Ma T, *et al*. 2009; Perfusion affects the tissue developmental patterns of human mesenchymal stem cells in 3D scaffolds. *J Cell Physiol* **219**: 421–429.

Zhu W, Chen J, Cong X, *et al*. 2006; Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Stem Cells* **24**: 416–425.

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