CLINICAL ORAL IMPLANTS RESEARCH

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Novel ceramic bone replacement material Osbone® in a comparative *in vitro* study with osteoblasts

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Abstract

Objective: Hydroxyapatite (HA) is a very common ceramic material for bone replacement due to its similarity in composition to the mineral phase of natural bone. A recently developed bone graft material is Osbone®, a synthetic HA ceramic available as porous granules with different sizes and block forms. The goal of this study was to characterise Osbone® *in vitro* in comparison to the already established calcium phosphate-based bone grafts Cerasorb M® and Bio-Oss®.

Materials and methods: Adhesion and proliferation of SaOS-2 osteoblasts were evaluated quantitatively by determining DNA content and lactate dehydrogenase (LDH) activity and qualitatively by scanning electron microscopy (SEM). In addition, MTT cell vitality staining was performed to confirm the attachment of viable cells to the different materials. Osteogenic differentiation of the cells was evaluated by means of alkaline phosphatase (ALP) activity quantification as well as by gene expression analysis of osteogenic markers using reverse transcriptase PCR.

Results: MTT staining after 1 day of adhesion showed viable cells on all examined materials. DNA content and LDH activity revealed proliferation of osteoblasts on Osbone® and Cerasorb M®, but not on Bio-Oss® during cultivation over 28 days. SEM showed a well-spread morphology of cells attached to both Osbone® and Cerasorb M®. We detected an increase of specific ALP activity during cultivation of osteoblasts on Osbone® and Cerasorb M® as well as expression of the bone-related genes ALP, osteonectin, osteopontin and bone sialoprotein II on both materials.

Conclusions: Osbone® granules support proliferation and osteogenic differentiation *in vitro* and are therefore promising candidates for *in vivo* applications.

Hydroxyapatite (HA)-based ceramic materials have been investigated extensively during the last decades and are widely used for bone replacement due to their osteoconductivity and high biocompatibility. In contrast to tricalciumphosphate (TCP) ceramics, which undergo faster resorption (Kamitakahara et al. 2008), HA ceramic materials are characterized by low resorbability (Porter et al. 2004; Mastrogiacomo et al. 2005). Osteoclastic action is necessary to resorb HA ceramic materials, whereas TCP ceramics dissolve physically with time. However, the slow resorption of HA ceramics may be advantageous for application in sites intended to support osseointegrated dental implants, because augmentation with fast resorbable TCP ceramics may lead to a loss of bone mass in these cases. Liljensten et al. (2003) demonstrated that both resorbable and nonresorbable HA ceramic materials promote new bone formation. Because the attachment of osteoblasts and their precursors as well as their proliferation at the surface of the bone graft material is a prerequisite to allow a good osseointegration, *in vitro* experiments are useful and necessary first steps for the evaluation of novel bone graft materials.

HA ceramics can be prepared by removing of organic components from natural sources such as bovine bone (Endobone®, Cerabone®) or algae (Algipore®). Another strategy to obtain HA ceramics is fully synthetic by sintering of HA powder. Osbone® was developed by curasan as a synthetic porous ceramic material from phase pure HA. In contrast to bone graft materials from xenogenic sources, synthetic ceramics bear no risk for transmission of pathogenic agents. Bio-Oss®, a deproteinized bovine bone material, which was heat treated at 300°C, is a HA material applied frequently in implant dentistry due to its close resemblance to natural spongious bone. However, some groups report the detection of residual proteins in Bio-Oss® material (Schwartz et al. 2000; Taylor et al. 2002), which might carry the risk of immunologic reactions. Other materials derived

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from bovine bone (Endobone® and Cerabone®) are highly sintered, and therefore, the presence of residual proteins is reliably excluded. However, due to the formation of CaO during the sintering process, the resulting HA ceramic can cause an alkaline pH in contact with liquids, which is not favourable for cellular attachment.

The goal of the present study is to characterize the *in vitro* performance of the newly developed fully synthetic HA material Osbone® in comparison to already established calcium phosphate bone graft materials. For this purpose, two materials were chosen, which have been successfully applied for bone defect reconstruction within the last years: Bio-Oss® comprises a HA material of bovine origin, and Cerasorb M® is a synthetic material from phase pure β-TCP. This study compares adhesion, proliferation and osteogenic differentiation of osteoblast-like cells on three different materials.

Granular materials are beneficial for application in surgical sites with complex shapes. With respect to further application *in vivo* and a good comparability, all *in vitro* examinations were performed with granular materials of a particle size of 1–2 mm. Hereby, the experimental parameters were adjusted to allow the evaluation of only those cells that were attached to the surface of the respective materials.

Material and methods

Materials applied in this study

Cerasorb M® granules (1–2 mm diameter) were prepared by curasan (Kleinostheim, Germany) as described by Peters & Reif (2004). Bio-Oss® (1–2 mm diameter) was purchased from Geistlich (Wollenhusen, Switzerland).

Osbone® granules were prepared by curasan as follows: powdered HA with phase purity ≥ 99% was purchased by VWR International (Darmstadt, Germany). The material was finely ground to a particle size of about 1–2 mm in average. A slurry of 65 weight % HA was prepared in a 1 : 1 water–isopropanol mixture under addition of the emulsifier Dolapix CE 64 (Zimmer & Schwartz, Lahnstein, Germany). Polyurethane (PU) foams, manufactured using the Schwartzwalder–Somers technique (Schwartzwalder 1963) (10 × 20 × 20 mm, 45 ppi–550 µm pore diameter and 80 ppi–300 µm pore diameter), were coated with the slurry. Excess slurry was removed by squeezing the sponge block. The PU foam was burned out in alumina crucibles using a sintering oven (Nabertherm N 100/H, Naber, Germany) at a temperature of 1000°C for 1 h. The HA particles in the slurry sinter together leaving the spongious structure of the burned-out foam. After the sintering process, the pore sizes were 250 µm and 100 µm, respectively. The spongious blocks were measured with X-ray powder diffractometry (STOE θ/θ diffractometer, Cu-$K\alpha$, $U = 40$ kV, $I = 35$ mA, 5–55°, 0.04°, 6 s) and showed a phase purity of 99%. The blocks were ground in a mortar to yield porous granules of a size of 1–2 mm. Granules with 100 and 250 µm pore size were mixed 1 : 1 to mimic a natural bone-like structure. The material was filled into glass vials, closed with a rubber stopper and crimped. The material was then sterilized by γ-radiation (≥ 25 kGy).

Cell seeding and cultivation of cell-seeded samples

SaOS-2 cells (ATCC 243, DSMZ, Braunschweig, Germany) were expanded in McCoy's 5A medium (Biochrom, Berlin, Germany), supplemented with 15% fetal calf serum, 10 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all from Biochrom) at 37°C and 5% CO_2.

Samples of 0.12 cm^3 of the different materials ($n = 5$ per time point and material for biochemical measurements, $n = 5$ per time point and material for gene expression analysis and some extra samples for microscopic evaluation) were seeded with 1.6×10^5 cells per sample. In some experiments, samples were pre-incubated for 24 h with a cell culture medium before seeding the cells. Next day, samples of cell-seeded granules were transferred to fresh 48-well plates and provided with fresh medium containing 5 mM β-glycerophosphate and 0.05 mM ascorbic acid-2-phosphate (both from Sigma-Aldrich, Taufkirchen, Germany). The medium was changed every 1–3 days, depending on the pH of the cell culture medium. Samples for biochemical measurements ($n = 5$) after 1, 7, 14, 21 and 28 days of cultivation were transferred to 1.5 ml test tubes after washing with PBS and frozen at − 80°C until further analysis. Samples for gene expression analysis ($n = 5$), taken after 1, 7, 14, 21 and 28 days of cultivation, were washed with PBS and subjected to RNA isolation as described below. Additional samples for SEM investigation were taken after 1 and 28 days of cultivation.

Determination of lactate dehydrogenase (LDH) activity, alkaline phosphatase (ALP) activity and DNA content

Frozen cell-seeded ceramic granules 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, each sample was sonicated for 10 s at 80 W with an ultrasonic processor UP 100 Hz (Dr Hielscher GmbH, Teltow, Germany).

One aliquot of the cell lysate was added to an ALP reaction buffer containing 1 mg/ml *p*-nitrophenyl phosphate (Sigma-Aldrich), 0.1 M diethanolamine, 1% Triton X-100 (pH 9.8), 1 mM $MgCl_2$, and the mixture was incubated at 37°C for 10 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 same cell lysate was mixed with an LDH reaction buffer (Cytotox96 kit, Promega, Madison, WI, USA). After incubation for 30 min at room temperature, 1 N acetic acid was added to stop the enzymatic reaction. Absorbance was measured at 492 nm.

Another aliquot of the cell lysate 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 using the multifunction microplate reader at an excitation and emission wavelength of 485/535 nm. Relative fluorescence units were correlated with the cell number using a calibration line.

Statistics

Data on biochemical measurements are represented by the means of five individual samples. Error bars represent standard deviation. Statistical comparisons were performed using a two-tailed, unpaired Student's *t*-test. A difference was considered significant at $P < 0.05$.

Scanning electron microscopy

Cell-seeded granules were washed twice with PBS, fixed for 60 min with 2% glutaraldehyde in PBS, washed with distilled water and dehydrated using a gradation series of ethanol/distilled water solutions. Critical point drying was performed with a CPD 030 apparatus (BAL-TEC, Balzers, Liechtenstein). Samples were mounted on stubs, coated with gold and imaged using a Philips XL 30/ESEM with FEG (field emission gun) operating in the SEM mode. The microscope was driven with an acceleration voltage of 5 kV and a working distance of 14 mm detecting secondary electrons.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining

After the cultivation of cell-seeded bone graft granules for 24 h, the cultures were supplemented with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) followed by further incubation at 37°C for 4 h. The formation of dark blue formazan dye

converted from MTT by mitochondrial dehydrogenases of living cells was documented using a stereomicroscope.

Reverse transcriptase PCR (RT-PCR)

Preparation of RNA from the cell-seeded granules cultivated for 1, 7, 14, 21 and 28 days, respectively, was performed using the peqGOLD MicroSpin Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions.

cDNA was transcribed from 250 ng of total RNA in a 20 µl reaction mixture containing 200 U of Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs (Invitrogen), 12.5 ng/µl random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U of RNase inhibitor RNase OUT (Invitrogen). For PCR experiments, 1 µl of cDNA was amplified in a 20 µl reaction mixture containing specific primer pairs to detect transcripts of ALP, osteonectin (ON), osteopontin (OP), bone sialoprotein II (BSPII) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, for each sample in a Thermocycler (Peqlab). The primers (MWG Biotech) and annealing temperatures for each gene 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 visualised using the FlashGel™ Dock system (Cambrex Bio Science, Rockland, NY, USA).

Table 1. Primers for RT-PCR

Markers	Primer sequences	$T_{annealing}$(°C)
ALP	Forward: 5′-ACCATTCCCACGTCTTCACATTTG-3′ Reverse: 5′-ATTCTCTCGTTCACCGCCCAC-3′	55
ON	Forward: 5′-ATCTTCCCTGTACACTGGCAGTTC-3′ Reverse: 5′-CCACTCATCCAGGGCGATGTAC-3′	57
OP	Forward: 5′-GTCTCAGGCCAGTTGCAGCC-3′ Reverse: 5′-GCCATGTGGCCACAGCATCTG-3′	59
BSPII	Forward: 5′-AATGAAAACGAAGAAAGCGAAG-3′ Reverse: 5′-ATCATAGCCATCGTAGCCTTGT-3′	55
GAPDH	Forward: 5′-GGTGAAGGTCGGAGTCAACGG-3′ Reverse: 5′-GGTCATGAGTCCTTCCACGAT-3′	55

ALP, alkaline phosphatase; BSPII, bone sialoprotein II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ON, osteonectin; OP, osteopontin, RT-PCR, reverse transcriptase PCR.

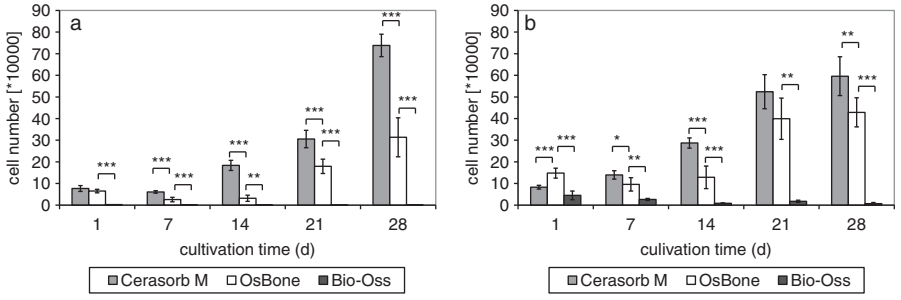

Fig. 1. Number of cells attached to Cerasorb M®, Osbone® and Bio-Oss® materials during cultivation for 4 weeks, $n = 5$ (± standard deviation of the mean). Cell number was calculated from LDH activity (a) as well as from DNA content (b) using a calibration line of known cell numbers. Significant differences between Cerasorb M® and Osbone® as well as between Osbone® and Bio-Oss®: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

Adhesion and proliferation of osteoblasts

The three different granule-shaped ceramic biomaterials were seeded with SaOS-2 osteoblasts and cultivated for up to 4 weeks. Proliferation of SaOS-2 cells on the different materials was evaluated with two assays: Determination of the DNA content covers the whole amount of attached cells, whereas measurement of the LDH activity reveals the presence of viable cells. In both cases, only cells that are attached to the material are included in the measurement; cells on the polystyrene culture dishes are not involved because the granules were removed from the culture dishes used for cell seeding and cultivation and washed before analysis.

When dry ceramic samples were seeded with cell suspension without prior incubation with the cell culture medium, we only detected a very low number of cells attached to Bio-Oss® granules, which diminished during further cultivation. In contrast, both Cerasorb M® as control and the novel material Osbone® supported the adhesion and proliferation of osteoblasts when seeded onto the dry samples (Fig. 1). Number of viable cells, determined from LDH activity, was in the same range as cell number calculated from the DNA content. On Bio-Oss® granules, the cell number calculated from the DNA content was higher compared with the number of viable cells from LDH activity measurement; however, there was no increase in the DNA content with cultivation time. Cell numbers on Cerasorb M® were significantly higher compared with that on Osbone® on days 7, 14 and 28 of cultivation; cell numbers on Osbone® were significantly higher compared with that on Bio-Oss® at all time points.

Proliferation of osteoblasts on Cerasorb M® and Osbone® granules was furthermore confirmed by SEM investigations of cell-seeded granules (Fig. 2a and b). Both materials were covered with a cell layer after 28 days of cultivation. In contrast, we were not able to detect attached cells on Bio-Oss® granules by SEM after the same cultivation time (Fig. 2c).

Because pre-treatment of biomaterials with serum-containing cell culture medium is a common practice to improve cellular adhesion, which is also recommended by some manufacturers of bone graft materials, we performed additional experiments involving pre-incubation of the ceramic granules with cell culture medium containing 15% FCS for 24 h. Cell-seeded samples were further cultivated for up to 2 weeks and quantitative biochemical analysis was again performed exclusively for cells that were attached to the biomaterials. We found a slightly higher amount of viable cells attached to Bio-Oss® granules compared with seeding of dry samples (Fig. 3). However, we did not detect an Increase of LDH activity during further cultivation. Determination of cell number from DNA content revealed a small increase of cell number on Bio-Oss®; nevertheless, we were not able to detect attached cells on this material after 2 weeks of cultivation by SEM (data not shown). On the other hand, pre-treatment with cell culture medium in the present set-up appears to be beneficial to the proliferation of osteoblasts on Cerasorb M® control and Osbone®. While the number of initially attached cells did not increase compared with seeding of dry samples, significantly higher cell numbers were detected on both materials during further cultivation compared with non pre-treated samples (significant differences in cell number calculated from the DNA content when dry samples are compared with pre-incubated samples: Cerasorb M® $P_{d7} = 0.00008$, $P_{d14} = 0.0019$, Osbone® $P_{d7} = 0.00022$, $P_{d14} = 0.00001$ [Fig. 1 compared with Fig. 3]).

Accordant to the quantified cell numbers, we were able to detect living cells on all three materials pre-incubated with cell culture medium after 24 h of initial adhesion using MTT staining (Fig. 4).

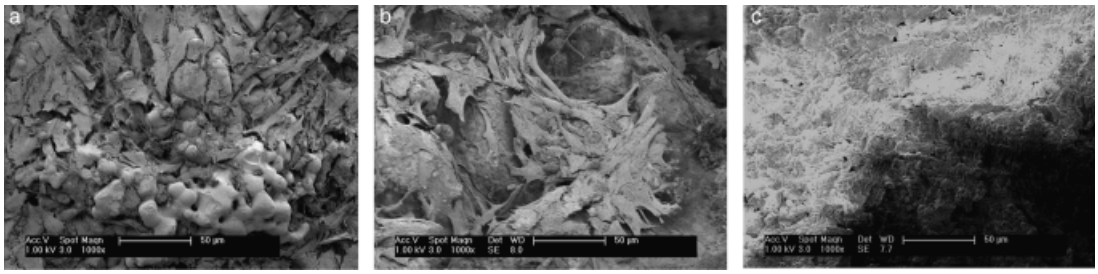

Fig. 2. SEM images of Cerasorb M® (a), Osbone® (b) and Bio-Oss® (c) granules seeded with osteoblasts after 28 days of cultivation.

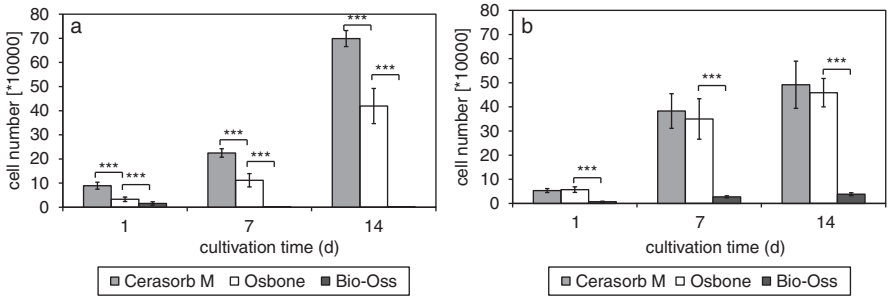

Fig. 3. Number of cells attached to Cerasorb M®, Osbone® and Bio-Oss® materials during cultivation for 2 weeks, all materials were pre-incubated with cell culture medium containing 15% FCS 24 h before cell seeding, $n = 5$ (+/− standard deviation of the mean). Cell number was calculated from lactate dehydrogenase activity (a) as well as from DNA content (b) using a calibration line of known cell numbers. Significant differences between Cerasorb M® and Osbone® as well as between Osbone® and Bio-Oss®: ***$P < 0.001$.

Moreover, we succeeded in detecting adherent cells on Bio-Oss® granules after 24 h of initial adhesion by SEM (Fig. 5). However, cells attached to Bio-Oss® after 24 h of adhesion showed a different morphology compared with cells on Cerasorb M® and Osbone® samples. On Cerasorb M® and Osbone® samples, we observed more stretched cells compared with Bio-Oss®.

Osteogenic differentiation

ALP activity

Because cell number on Bio-Oss® samples was very low even after pre-treatment with cell culture medium, investigations of osteogenic differentiation were performed only on Cerasorb M® as control and Osbone®. ALP activity was determined for osteoblasts seeded onto dry as well as on pre-incubated ceramic samples. We found an increase of specific ALP activity (ALP activity related to cell number) for both dry and pre-incubated samples on both materials (Fig. 6). At all examined time points of cultivation, specific ALP activity was significantly higher for pre-treated samples compared with dry samples (Cerasorb M®: $P_{d1} = 0.0009$, $P_{d7} = 0.000005$, $P_{d14} = 0.00053$; Osbone®: $P_{d1} = 0.00003$, $P_{d7} = 0.00049$, $P_{d14} = 0.0011$).

Moreover, specific ALP activity was significantly higher on Cerasorb M® at day 21 and day 28 of cultivation in the case of dry samples and at day 7 of cultivation in the case of pre-incubated samples (Fig. 6).

Gene expression of osteogenic markers

Gene expression of the osteogenic markers ALP, ON, OP and BSPII was proven for SaOS-2 cells on both Cerasorb M® and Osbone® granules without medium pre-incubation of the ceramic samples. Gene expression of osteogenic markers on Bio-Oss® was not analysed because it was not possible to extract a sufficient amount of RNA due to the very low number of attached cells. There were no remarkable differences in gene expression on Cerasorb M® control and Osbone®, indicating that both materials support osteogenic differentiation (Fig. 7).

Discussion

HA ceramics have been widely used as implant materials for bone regeneration, mainly due to their similarity in composition to the mineral phase of natural bone. Numerous reports proved the high biocompatibility of HA ceramics as well as their potential to facilitate new bone formation *in vivo* (Yoshikawa et al. 1996; Ayers et al. 1999; Dong et al. 2001; Huber et al. 2008). Furthermore, different *in vitro* studies revealed that porous HA ceramics are excellent scaffolds for the proliferation and osteogenic differentiation of osteoprogenitor cells (Nordström et al. 1999; Okamoto et al. 2006; Scaglione et al. 2006; Mygind et al. 2007; Matsushima et al. 2009). HA-based Osbone® granules, applied in the present study, revealed very good osteoblast attachment, proliferation and osteogenic differentiation. In contrast, we detected only few attached osteoblasts on Bio-Oss® granules at early time points of cultivation and we were not able to demonstrate osteoblast proliferation on this material over longer cultivation times *in vitro*. Different sintering and manufacturing parameters of HA-based ceramic materials can affect considerably physical properties such as solubility and probably also biological properties (Weibrich et al. 1999). Therefore, it is not surprising that different HA materials, such as Bio-Oss® and Osbone®, show a completely different behaviour with regard to cellular adhesion, proliferation and osteogenic differentiation. Comparing the β-TCP-based ceramic Cerasorb M® and Bio-Oss®, we also found strongly significant differences in cell viability in all conducted experiments (Figs 1 and 3). This is in concordance with a study by Herten et al. (2009), who cultivated primary osteoblasts on different granule-shaped bone graft materials. When seeding the cells on Bio-Oss® granules, only after 2 h of cultivation, a small number of viable cells were detectable on the material and the viable cell number decreased during further cultivation. In contrast, the number of viable cells increased steadily over 14 days of cultivation when cells were cultivated on Cerasorb M® granules (Herten et al. 2009). Kübler et al. (2004) detected the lowest level of viable cells on Bio-Oss® compared with four other granule-shaped bone graft materials and did not find an increase in viability during further cultivation. Handschel et al. (2009) cultivated murine MSC on five different biomaterials including Bio-Oss® and Cerasorb M® and detected the lowest cell number by far on Bio-Oss®. Turhani et al. (2005) observed that the growth of human mandibular osteoblasts in the presence of Bio-Oss® granules was significantly lower than in the presence of other HA-based bone substitutes. Poor cell adhesion on Bio-Oss® was also reported by Asti et al. (2008), who investigated SaOS-2 cells on Bio-Oss® scaffolds after 15 days of cultivation. Using SEM, they detected only

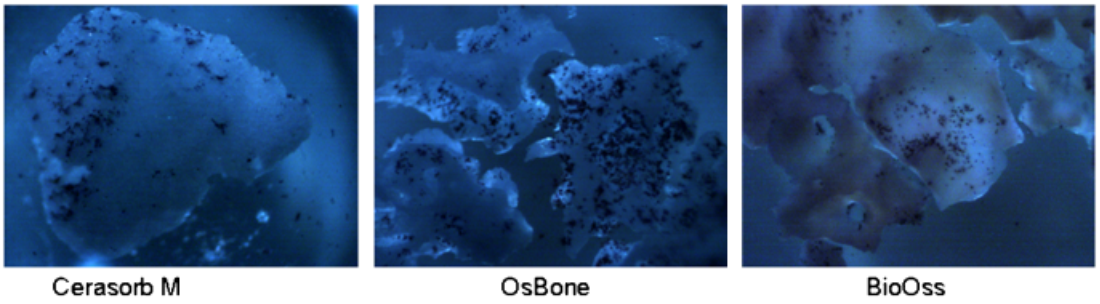

Fig. 4. MTT staining of Cerasorb M®, Osbone® and Bio-Oss®, pre-incubated with cell culture medium and seeded with osteoblasts after 1 day of cell adhesion, stereomicroscopic images, magnification × 20.

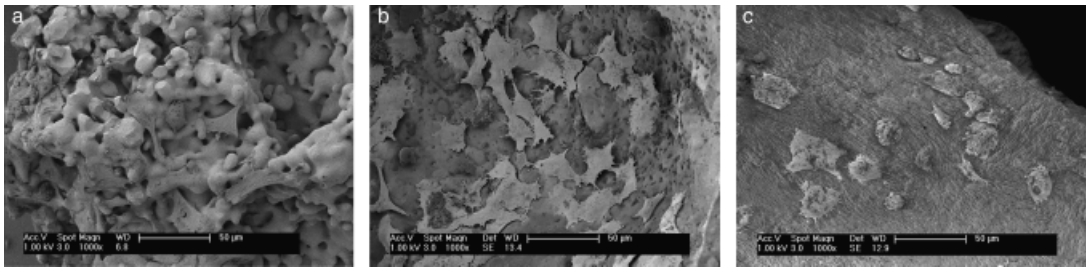

Fig. 5. Scanning electron microscope images of Cerasorb M® (a), Osbone® (b) and Bio-Oss® (c) granules, pre-incubated with cell culture medium and seeded with osteoblasts after 1 day of cell adhesion, magnification ×1000.

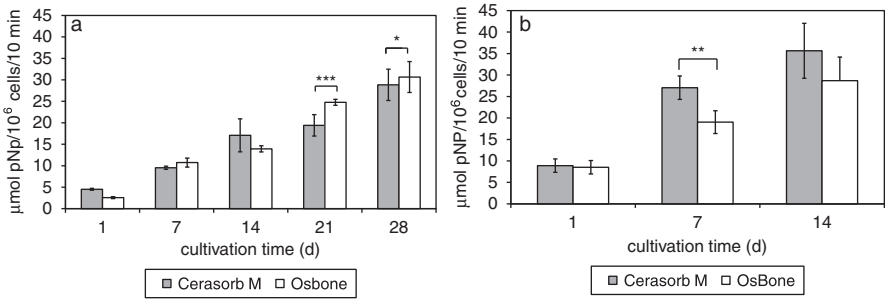

Fig. 6. Specific alkaline phosphatase (ALP) activity of SaOS-2 osteoblasts grown on Cerasorb M® and Osbone® granules, $n = 5$ (+/− standard deviation of the mean). ALP activity was related to cell number determined by DNA measurement. (a) Dry samples were seeded with cells, (b) samples were pre-incubated with cell culture medium before cell seeding. Significant differences between Cerasorb M® and Osbone®: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

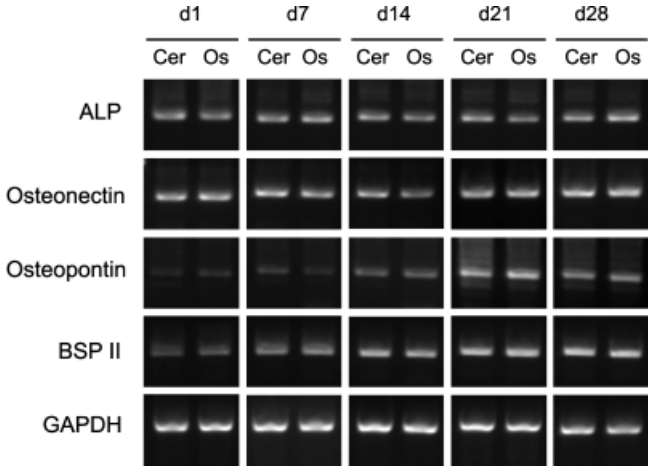

Fig. 7. Gene expression of alkaline phosphatase (ALP), osteonectin, osteopontin, bone sialoprotein II (BSPII) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for SaOS-2 osteoblasts seeded on Cerasorb M® and Osbone® granules after 1, 7, 14, 21 and 28 days of cultivation. RNA of five cell-seeded samples was combined for each analysis. GAPDH was used as an internal control.

sporadically attached cells, which were less spread out over the surface. Interestingly, coating of Bio-Oss® with polylactide resulted in considerably better cell adhesion. Recently, an interesting study on the initial adhesion of osteoblasts to Bio-Oss® granules was published (Rameis et al. 2009). The investigation involved liquid scintillation counting of radiolabelled cells as well as viability and morphological studies and revealed a relatively low amount of adherent cells. Furthermore, attached cells appeared only occasionally in SEM investigations, which is in agreement to our findings (Fig. 5). Nevertheless, Bio-Oss® is a well-established bone graft material, which has been successfully applied in implant dentistry, especially in sinus floor augmentation procedures and it has been documented that Bio-Oss® promotes osteogenesis *in vivo* (Tapety et al. 2004; Orsini et al. 2007; Abushahba et al. 2008; Cordaro et al. 2008). Besides a plethora of studies on Bio-Oss® describing the excellent *in vivo* performance of this bone graft material, there are also a number of studies showing that Bio-Oss® delays and/or obstructs bone formation *in vivo* (Beloti et al. 2008; Araújo & Lindhe 2009). Cerasorb M® is less common than Bio-Oss® in bone defect reconstruction; however, there are various studies underlining its suitability for bone replacement and its potential to facilitate new bone formation (Szabó et al. 2001; Palti & Hoch 2002; Horch et al. 2006; Suba et al. 2006). The well-documented advantage of Cerasorb M® in comparison with Bio-Oss® *in vitro* is not reflected *in vivo*. Comparative *in vivo* studies

involving both materials revealed a similar clinical performance (Mangano et al. 2007; Simunek et al. 2008), with Bio-Oss® showing even more new bone formation compared with Cerasorb M®. Therefore, *in vitro* investigations of biomaterials cannot completely replace animal experiments and clinical studies; however, those studies are a useful tool for first biocompatibility studies of newly developed materials and may reduce the demand for animal experiments, which are more cost intensive and always cause ethical concerns, especially at an early stage of material development. Further advantages of *in vitro* studies are the possibility to quantify cellular behaviour like adhesion, proliferation and differentiation. Visualization of cell morphology is possible on the electron microscopic level. *In vitro* studies are advantageous with regard to a defined environment for the study of cell–material interactions, which is not influenced by specific characteristics of individual patients or animals.

In the present cell culture study, Osbone® turned out to be a suitable substrate, which supports adhesion, proliferation and osteogenic differentiation of osteoblasts. *In vitro* studies involving granule-shaped biomaterials often perform the cultivation of cells only in the presence of the respective biomaterial (Rice et al. 2003; Aybar et al. 2004; Kübler et al. 2004; Le Nihouannen et al. 2007; Herten et al. 2009). In our study, we chose experimental parameters that allowed the quantitative evaluation of exclusively such cells, which adhered to the surface of the material itself. Cells that adhered and proliferated on the plastic surface of the cell culture test plates were completely excluded. In the present experimental setup, Osbone® revealed a significantly higher number of attached cells compared with Bio-Oss® at all time points of investigation (Figs 1 and 3). Although proliferation rates of osteoblasts on Osbone® granules were lower compared with Cerasorb M® granules (Figs 1 and 3), expression of osteogenic markers was in a similar range for Osbone® and Cerasorb M® (Fig. 7), suggesting the potential of Osbone® for applications *in vivo*.

Conclusions

The novel HA ceramic bone replacement material Osbone® supports adhesion, proliferation and osteogenic differentiation of SaOS-2 osteoblasts. Morphology and number of attached cells as well as expression of bone-related markers are comparable to the already established β-TCP ceramic Cerasorb M®. In contrast, Bio-Oss® granules were not able to support the proliferation of osteoblasts in the present *in vitro* study. Because of the good *in vitro* performance of Osbone®, this material is a promising candidate for application *in vivo*.

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