VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 70, No. 3, pp. 154–160

ISSN 0015–5659
www.fm.viamedica.pl

Biocompatibility of bone graft substitutes: effects on survival and proliferation of porcine multilineage stem cells *in vitro*

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[Received 24 February 2011; Accepted 8 May 2011]

Bone graft substitutes (BGS) are widely used in clinical practice. For stem cell-based approaches to bone tissue engineering BGS need to show sufficient biocompatibility in the in vitro *setting. This study was designed to demonstrate the influence of six different BGS on the proliferation and metabolic activity of porcine mesenchymal multilineage stem cells (pMSC)* in vitro.

Bone-marrow derived pMSC were cultivated for 24 hours with the eluates of six different BGS. The eluates were generated by incubating the BGS three times in succession for 24 hours with a culture medium and collecting the supernatants. pMSC vitality and proliferation in the presence of eluates from the first, second, and third incubation were assessed by WST-test quantification of metabolically active cells.

Culture of pMSC with eluates in all cases resulted in decreased cell numbers in an eluate concentration-dependent manner. At least a 65% loss of cells compared to controls (culture medium without eluates) could be observed in the presence of undiluted eluates. The negative influence of eluates varied significantly among BGS. In all cases, second and third eluates were less potent in their negative effects on cellular vitality/proliferation.

In conclusion, the BGS examined here should be submitted to thorough preincubation before in vitro *use for cell-based constructs to maximize cell viability for the tissue engineering of bone.* (Folia Morphol 2011; 70, 3: 154–160)

Key words: bone tissue engineering, cell viability, cytotoxicity, mesenchymal stem cells

INTRODUCTION

Large skeletal defects require bone grafts for regeneration. The availability of autografts may be limited and associated with significant donor site morbidity [19, 45, 46]. Allografts bear the risk of immunological rejection and disease transmission [22]; therefore, bone graft substitutes (BGS) are widely used in clinical practice. They include hydroxyapatite and beta-tricalcium phosphate ceramics [11, 40], demineralised bone composites [5, 32], and polymers consisting of polyglycolic and polylactic acids [24]. Bone graft substitutes should maintain spatial integrity, provide biomechanical stability, allow for cell attachment, vascular ingrowth, and bone deposition, and should be degradable, ultimately leading to complete replacement by bone [6].

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Table 1. Characteristics of bone graft substitutes with good biocompatibility in the *in vivo* setting

Brand name	Material	Granule size [mm]	Porosity [%]	Pore size [μm]
BioOss®	Deproteinised bovine bone	1–2		
Straumann Bone Ceramic®	Synthetic, 60% HA, 40% β-TCP	0.5–1.0	90	100–500
Cerasorb®	Synthetic, > 99% β-TCP sintered at > 1000°C	0.5–1.0	30 ± 5	0.1–50
NanoBone®	Synthetic, 76% HA, 24% SiO_2 manufactured at 700°C	0.6	> 80	0.01–0.02
NovaBone-OM®	Synthetic, Si, Na, Ca, P, O	0.09–1.0		
Pepgen P15®	Bovine HA coated with peptide (= active domain of collagen I)	0.25–0.42		

HA — hydroxyapatite; β-TCP — β-tricalcium phosphate

The effectiveness of BGS may be enhanced by combining appropriate scaffolds with osteogenic cells [34].

Since the discovery and isolation of bone-forming cells from adult bone marrow by Friedenstein and colleagues [8, 9, 26], who referred to them as colony forming unit fibroblasts (CFU-F) or bone marrow stromal stem cells (BMSC), research has focussed on cultivating and characterising these stem cells *in vitro* [4]. Their multilineage potential has been demonstrated in humans [28] and other species [3, 30].

Multilineage stem cells have proven to be an excellent source of osteogenic cells. When combined with BGS these stem cells may enhance bone regeneration *in vivo* [4, 10, 16, 35]. For tissue engineering purposes, i.e. the *in vitro* cultivation of cell-seeded constructs, scaffold materials should support multilineage stem cells viability to minimise the amount of donor cells needed while providing high numbers of functional osteogenic cells.

Most studies on the biocompatibility of BGS were designed as seeding experiments [23, 37] and have used continuous cells lines [25, 38, 41], osteoblasts [1, 18], fibroblasts [15] or other non-osteogenic cells [43]. In addition, most studies deal with the testing of single BGS rendering direct comparison between different BGS impossible. Finally, information on the early effects of BGS on cell metabolism is scarce.

This study was designed to demonstrate and compare the early effects of six different BGS on the proliferation and metabolic activity of porcine mesenchymal multilineage stem cells (pMSC) *in vitro*.

MATERIAL AND METHODS

This study was approved by the Minister of Nature, Environment, and Forestry of Schleswig-Holstein and was in accordance with the local ethics committee (V 742-72241.121-1415-2/04).

Bone graft substitutes

Six different types of sterile BGS of diverse chemical and morphological type were purchased and stored at room temperature (Table 1). They were biologically derived or synthetic granular materials typically found in current clinical practice: BioOss® (Geistlich, Wolhusen, Switzerland) [12, 13, 48], Straumann Bone Ceramic® (Straumann AG, Freiburg, Germany), Cerasorb® (Curasan AG, Kleinostheim, Germany) [27], NanoBone® (Artoss GmbH, Rostock, Germany), NovaBone-OM® (NovaBone Products, Alachua, Fl, USA), and Pepgen P15® (Friadent GmbH, Mannheim, Germany) [25, 29, 39].

Generation of BGS eluates

Material properties of BGS, such as particle size, surface morphology, electric charge, porosity, and pore size, may significantly influence cell adherence [7, 17, 21], proliferation, and phenotype [47], making direct comparison between the various BGS difficult. In contrast to common seeding experiments, BGS eluates were used to reduce the complexity of *in vitro* culture conditions and render comparison among the various BGS possible. All experiments were conducted in triplicate.

BGS eluates were generated by incubating 0.1 mL of each BGS three times in succession for 24 h with 1 mL of culture medium (CM) at 37°C and collecting the supernatants. This time period of 72 h was chosen to evaluate the early effects of soluble factors released from the BGS as well as to examine time dependent effects. The resulting eluates of the first, second, and third incubation were stored at 4°C until further use, to cultivate pMSC (see below).

Cell source and cell culture

Multilineage stem cells were harvested from the iliac bone marrow of an adult (23 months old) Göttingen minipig (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark), as previously described [20]. Briefly, under general anaesthesia with ketamine and xylazine and aseptic conditions, approximately 1.5 mL of spongious bone were harvested, dissected into small fragments of 0.1–0.5 cm, and pretreated with 0.1% collagenase (Nordmark, Uetersen, Germany) for 2 h at 37°C. After thoroughly rinsing with PBS (pH = 7.4) to remove collagenase solution, the cells were stained with 4% trypan blue (Gibco, Paisly, Scottland, UK), counted in a haemocytometer, and plated at a density of 5,000 cells/ /cm^2 in T75 tissue culture flasks (passage 0). The CM consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with 10^5 IU/L penicillin, 100 mg/L streptomycin (all Biochrom AG, Berlin, Germany), 1 mM L-ascorbic acid (Sigma, Deisenhofen, Germany), and 10% FCS. Cells were cultivated under standard conditions at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed first after 24 h to remove non-adherent cells, and twice per week thereafter. At 70–80% confluence (on day 14), cells were detached under microscopic control after washing with D-PBS (Gibco, Paisly, Scottland, UK) for 2 min followed by incubation with Accutase (PAA, Cölbe, Germany) for 15 min at 37°C. Detached cells were suspended in CM, centrifuged for 5 min at 1,200 rpm, re-suspended in CM, stained with trypan blue, counted as above, replated at a density of 2,500 cells/cm^2 (passage 1), and cultivated until subconfluent (70–80%) again. Subcultivation was further continued to passage 3 as described. This allowed for standardisation because the same cell line was used for all experiments.

Characterisation of pMSC

Bone marrow-derived porcine cells were characterised for their multilineage potential by cultivation with osteogenic and adipogenic induction media. Briefly the cells were seeded in 6-well plates at a density of 5,000 cells/cm^2 and cultivated with CM for 7 days until subconfluent. For osteogenic or adipogenic induction, cultivation was continued with either osteogenic or adipogenic induction medium for 14 days. Osteogenic induction medium consisted of CM supplemented with 100 nM dexamethasone, 10 mM beta-glycerol phosphate, 230 mg/L $CaCl_2$, and 52 mg/L L-ascorbic acid (all Sigma, Deisenhofen, Germany). Adipogenic induction medium (Adipogenic Differentiation Media BulletKits, Cambrex Bio Science, Walkersville, MD, USA) was used according to the manufacturer's instructions. Cells cultivated for 14 days with CM but without induction factors served as controls. All media were changed twice per week. After 14 days the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 10 min, and rinsed again with PBS. Osteogenic or adipogenic differentiation was evaluated after staining with Alizarin Red S or Oil Red O, respectively, by microscopic examination of the presence of extracellular calcium complexes or intracellular fat vesicles, respectively.

Biocompatibility testing

Expanded subconfluent pMSC of passage 3 were seeded in duplicate on 96-well plates at a density of 5,000 cells per well (samples) in 100 μL of CM and allowed to adhere to the plate. A duplicate standard row of 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312, and 156 cells per well in 100 μL of CM served to establish a standard curve (standard) on each plate. Wells with 100 μL CM but without cells served as negative controls (blanks). Identical homogenous distribution of cells throughout the sample wells was confirmed by microscopic examination after 24 h of cultivation.

Adherent cells were then cultivated with previously generated first, second, and third incubation eluates of the six different BGS (see above). To study concentration dependent effects, eluates were used undiluted (100%) or diluted with CM at a ratio of 1:1 (50%), 1:3 (25%), or 1:7 (12.5%). Wells cultivated with CM only (i.e. 0% eluates) served as controls.

After 24 h of eluate culture pMSC viability and proliferation were assessed by quantification of metabolically active cells using the WST-1 test (Roche Diagnostics GmbH, Mannheim, Germany) [2]. According to the manufacturer's instruction the medium was replaced by a 10% WST-1 solution. After 45 min of incubation at 37°C, WST-1 cleavage and formazan formation by metabolically active cells was measured photometrically at 450 nm with an ELISA 96-well plate reader (SPECTRAmax® PLUS, Molecular Devices, Sunnyvale, CA, USA). Six measurements per BGS and type of eluate were recorded.

Statistical evaluation

Statistical analysis consisted of a two-factorial analysis of variance for repeated measures of extinction values. The significance level was set at $p < 0.05$.

RESULTS

Porcine bone marrow-derived cells showed multilineage potential after 14 days of osteogenic or adipogenic induction as demonstrated by the pres-

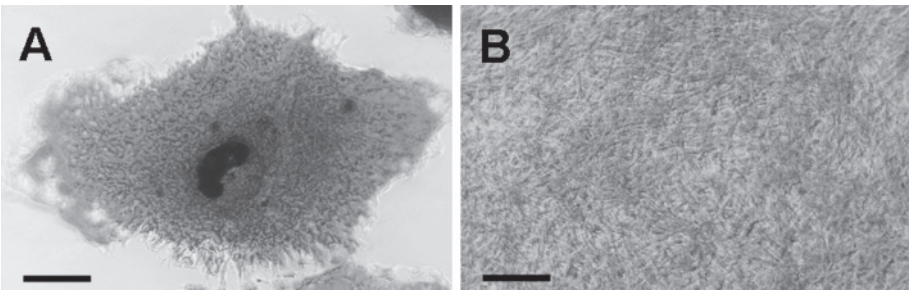

Figure 1. Phase contrast microscopy of pMSC cultivated for 14 days with (**A**) or without (**B**) osteogenic supplements and stained with Alizarin Red S. Osteogenic potential of induced cells is indicated by the presence of extracellular calcium complexes; bar = 200 μm.

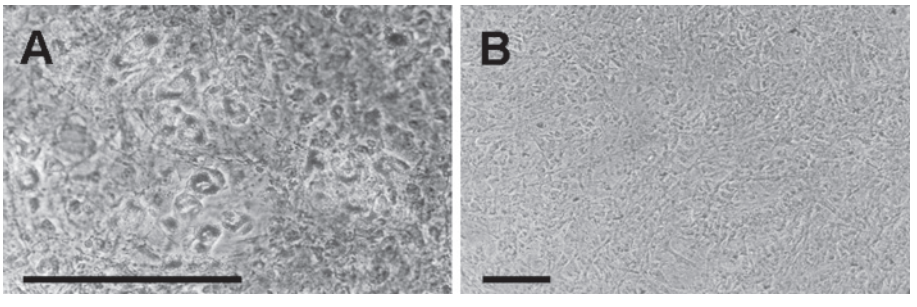

Figure 2. Phase contrast microscopy of pMSC cultivated for 14 days with (**A**) or without (**B**) adipogenic supplements and stained with Oil Red O. Adipogenic potential of induced cells is indicated by the presence of intracellular fat vesicles; bar = 200 μm.

ence of extracellular calcium complexes or intracellular lipid vesicles, respectively. Cells cultured without inductions factors (controls) did not form calcium complexes or intracellular lipid vesicles (Figs. 1, 2).

Culture of pMSC with BGS eluates resulted in significantly decreased cell numbers in all cases. At least a 65% loss of cells compared to controls (CM without eluates) was observed in the presence of undiluted eluates (Fig. 3A). The differences between undiluted eluates and 50%-eluates were significant ($p < 0.001$), as were the differences between 50%, 25%, and 12.5%-eluates ($p < 0.05$).

The negative influence of eluates from the various BGS differed. For first incubation eluates, BioOss® decreased vitality most and NovaBone-OM® least. The others ranged in between in the following order: BioOss® < NanoBone® < Straumann Bone Ceramic® < Cerasorb® < Pepgen P15® < NovaBone-OM® ($p < 0.05$). For second and third incubation eluates, the differences between BGS were similar but not statistically significant. Independent of the type of BGS, dilution of eluates resulted in an increase of metabolically active cells ($p < 0.001$). In all cases, second and third eluates were less potent in their effect on cellular vitality/proliferation ($p < 0.01$) (Fig. 3B).

DISCUSSION

In all cases BGS eluates had a negative effect on cell survival and proliferative activity, resulting in significantly reduced cell numbers when compared to controls. At most, only one third of adherent cells survived when cultivated with BGS eluates for 24 h. This may be explained by a toxic effect eluted from the BGS, such as changes in pH-value seen with synthetic hydroxyapatites [31]. Jäger et al. [14] reported extensive H+ release by BGS incubated in DMEM (pH 7.4–7.6) for 24 h. They found significant differences in H+ concentrations between the tested materials. While Cerasorb® led to an increase in pH to 8.09, non-demineralized bovine bone (Lubboc™, Ost, Developpement, France) and demineralized human bone caused a decrease in pH to 7.3 and 6.66, respectively. Cell survival after 4 days of eluate culture was higher for Cerasorb® than for non-demineralized bovine bone or decalcified human bone, which is consistent with the findings of this study

Kauschke et al. [15] used the Alamar Blue assay to measure metabolic activity of mouse and human fibroblasts during 28 days and 24 days of culture with Straumann Bone Ceramic® and NanoBone®. They demonstrated equal cytotoxicity of these two BGS, which is in accordance with the neighbouring

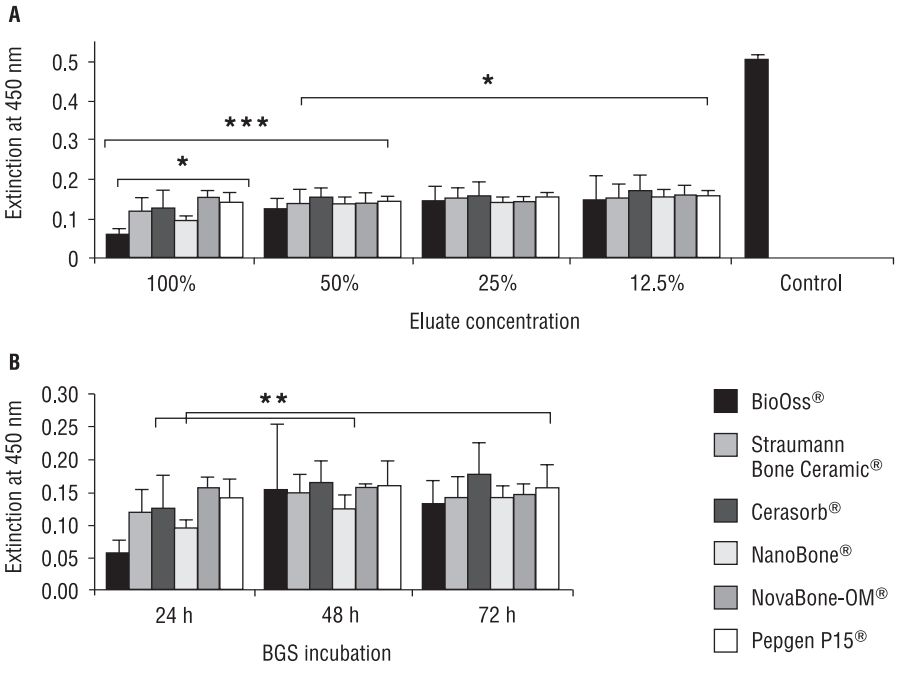

Figure 3. Results of WST-1 test after cultivation of pMSC with eluates of six different bone graft substitutes (BGS); **A.** At all concentrations, cultivation of pMSC with first incubation eluates (24 h) resulted in significantly reduced cell numbers of max. 35% of controls (culture medium without BGS); differences in cell numbers among BGS cultivated with undiluted first incubation eluates were significant; **B.** Second and third BGS eluates resulted in a significant increase in cell numbers; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ranking positions shown here. Viability on day 2 of culture was about 50% and 60%, respectively, of control cultures (cells without BGS) for the two materials. The authors attribute the reduction of metabolic activity to BGS surface characteristics. In this study, in which surface characteristics were unimportant, cell viability after 24 hours of culture with BGS eluates was reduced to about 15% of controls, and a concentration dependent effect could be observed for all materials including NanoBone®.

Trasatti et al. [39] measured higher concentrations of TGF-β1 in supernatants of rat osteoblasts when cultured with Pepgen P15® compared to BioOss®. Nguyen et al. [25] reported twofold higher cell viability by MTT-assay of human osteosarcoma cells 4 days after seeding onto anorganic bovine bone matrix coated with the P15-peptide compared to P15--peptide-free controls. Kübler et al. [18] used the WST-1 test to assess the viability of human osteoblasts after 6 and 9 days of culture with five different BGS, including BioOss® and Pepgen P15®. While cell proliferation and viability of Pepgen P15® cultures were equal or higher than control cultures without BGS, values for BioOss® cultures were lowest of all BGS tested, amounting to about one third of control values. Wiedmann et al. [44] measured the viability of human osteoblast-like cells cultivated for one week on 16 different biomaterials, including collagen, tricalcium phosphates, hyaluronic acid, silicone, and anorganic bovine bone (BioOss®), using the EZ4U assay. While none of the materials reached control values, cells cultured with BioOss® showed the lowest metabolic activity (~20%) of controls. Turhani et al. [42] measured total DNA content from human osteoblasts after 6 days of culture on Pepgen P15® and BioOss® using the PicoGreen assay. While values in Pepgen P15® cultures were not significantly lower than controls, DNA content in BioOss® cultures was less than 10% of controls. All these findings are consistent with the results of this study, in which eluates of Pepgen P15® led to a higher number of metabolically active cells than eluates of BioOss®.

Apart from H+ release, substances needed during manufacturing (e.g. glycerol for storage, acid for decalcification) or the BGS itself (residual mineral) may be responsible for cell death in *in vitro* assays.

Due to limited resources of BGS and pMSC we were unable to repeat the experiments and analyse eluate composition or pH. Theoretically it is conceivable that, apart from toxic substances being released from BGS into the eluate during incubation, factors within the medium, which enhance cell viability and proliferation,

e.g. growth factors, could be removed from the eluate by binding to the BGS during incubation. During second and third incubation, with binding sites being increasingly occupied, their relative concentration in the eluate would increase, which could account for higher cell numbers in second and third incubation eluates. Also, by diluting eluates with CM, the concentration of "favourable factors" would increase through the addition of CM. It has been shown that binding proteins to BGS surfaces enhances adherence and proliferation [33, 36]. To test this hypothesis, seeding experiments with the BGS and pMSC of this study were conducted [47]. Cell adherence and proliferation significantly depended on the type of BGS, and cell densities at different time points paralleled the results of the present study. Therefore, the above hypothesis of a "steal effect" can be rejected.

While the BGS tested here have shown good biocompatibility in clinical trials, *in vitro* conditions differ from the *in vivo* setting. Thus, the results of this study cannot be transferred to the *in vivo* situation. Since the concept of tissue engineering involves the combination of biomaterials, cells, and growth factors *in vitro*, i.e. the cultivation of seeded constructs for *in vivo* implantation, there is a need to study the *in vitro* effects of BGS for tissue engineering applications.

CONCLUSIONS

In conclusion, the eluates of the BGS tested here had a negative effect on cell survival of pMSC cultured *in vitro*. The negative influence exerted by second and third eluates was significantly lower. Thus, BGS should be submitted to (repeated) preincubation washes prior to cell seeding, in order to ascertain cell viability and a solid proliferative response of mesenchymal multilineage stem cells *in vitro*. Future studies should be directed towards optimising cell viability *in vitro* for the applications of engineered constructs *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Federal Ministry of Economics and Labour, Grant # KF 0007001 UL7.

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