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Efficacy of bioreactor-activated bone substitute with bone marrow nuclear cells on fusion rate and fusion mass microarchitecture in sheep

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Abstract

Bioreactors have been used for bone graft engineering in pre-clinical investigations over the past 15 years. The ability of bioreactor-incubated bone marrow nuclear cells (BMNCs) to enhance bone-forming potential varies significantly, and the three-dimensional (3D) distribution of BMNCs within the scaffold is largely unknown. The aims of this study were 1) to investigate the efficacy of a carbonated hydroxyapatite (CHA) with/without BMNCs on spine fusion rate and fusion mass microarchitecture using a highly challenging two-level posterolateral spine fusion without instrumentation; and 2) to evaluate 3D distribution of BMNCs within scaffolds characterized by immunohistochemistry. Fusion rate and fusion mass were quantified by micro-CT, microarchitectural analysis and histology. While the homogenous 3D distribution of BMNCs was not observed, BMNCs were found to migrate towards a substitute core. In the autograft group, the healing rate was 83.3%, irrespective of the presence of BMNCs. In the CHA group, also 83.3% was fused in the presence of BMNCs, and 66.7% fused without BMNCs. A significant decrease in the fusion mass porosity (p=0.001) of the CHA group suggested the deposition of mineralized bone. The autograft group revealed more bone, thicker trabeculae, and better trabecular orientation but less connection compared to the CHA group. Immunohistochemistry confirmed the ability of bioreactors to incubate a large-sized substitute coated with viable BMNCs with the potential for proliferation and differentiation. These findings suggested that a bioreactor-activated substitute is comparable to autograft on spine fusion and that new functional bone regeneration could be achieved by a combination of BMNCs, biomaterials, and bioreactors.

Keywords: Flow perfusion bioreactor, spinal fusion, bone graft substitute, bone marrow nuclear cells, bone microarchitecture, immunohistochemistry
INTRODUCTION

A bone graft is one of the most frequently transplanted tissues – second only to a blood transfusion – with over two million bone graft procedures being performed annually worldwide (Campana et al., 2014). The gold standard autograft and allograft are most commonly used since their bioactive properties are in favor of bone formation. However, problems such as limited availability and donor site morbidity have led to the increased use of synthetic bone grafts. Supplementing grafts with stem cells considerably improves the feasibility since the majority of grafts have osteoconductive property only (Marcacci et al., 2007). To date, engineered synthetic bone grafts suitable for bone replacement in bone defects of clinically relevant sized remain a problem to be fully solved, as they have reduced bioactivity when compared to autograft/allograft (Cancedda, Giannoni, & Mastrogiacomo, 2007; Evans, Davies, Dare, & Oreffo, 2013). Therefore, the grafting procedure using a synthetic bone graft has not been introduced as a qualified standardized treatment or supplement to conventional management strategies.

A major challenge in engineering cell-based bone grafts is that only approximately 0.015% of the human mononuclear cells can form a colony-forming unit–fibroblast (CFU-f). This potential declines with donor age along with the proliferative capacity and the osteogenic potential of bone marrow nuclear cells (BMNCs) (Mageed, Pietryga, DeHeer, & West, 2007). In order to increase the number of BMNCs followed by more CFU-f, mononuclear cells are most commonly expanded in vitro using the adherence technique. One problem with this approach is that human BMNCs are known to have limited proliferative capacity and lose their characteristics after intensive in vivo expansion (Banfi et al., 2002). They reach replicative senescence after 24 - 40 population doublings, depending on donor age (Bruder, Jaiswal, & Haynesworth, 1997). Hence, 2D in vitro culturing, expansion of the BMNCs before seeding on to substitutes, has resulted in low seeding efficiency (Izadpanah et al., 2008).
Notably, the bioreactor culture of BMNCs directly onto the bone graft substitute matrix has been suggested as a qualified alternative to the conventional 2D plate culture (Peroglio, Gaspar, Zeugolis, & Alini, 2018). By culturing tissues in a sterile \textit{in vitro} environment mimicking \textit{in vivo} conditions, bioreactors have been suggested to influence the seeding efficiency and expansion/differentiation of the progenitor cells as well as the survival and homogenous distribution of osteoprogenitor cells, which positively results in functional viable tissues for implantation (Braccini et al., 2005).

A critical issue with the bioreactor culture of larger-sized substitutes is the insufficient penetration of osteoprogenitor cells into the substitute. Therefore, non-homogenous cell distribution results in insufficient vascularization and central substitute necrosis. Therefore, in the assessment of bioreactor effects on bone formation, it remains important to characterize the cells within the substitute with respect to bone-forming potential after seeding prior to \textit{in vivo} implantation.

The purposes of this study were 1) to evaluate the effects of a bioreactor-activated large-sized carbonated hydroxyapatite (CHA) bone graft substitute compared to autograft on fusion rate and the microarchitecture of fusion mass in a two-level posterolateral lumbar spine fusion (PLF) without instrumentation in sheep, and 2) to assess the seeding efficiency of bone graft substitute and characterize BMNCs after bioreactor activation by immunohistochemistry to explore the efficacy of bioreactor in further details.

We hypothesized that the fusion efficacy and microarchitecture of spine fusion mass in the CHA group would be similar to those of the autograft group, and that the BMNCs would have bone-forming potential after bioreactor activation.

2 | MATERIALS AND METHODS

2.1 Study design
This study includes two parts: *in vivo* spine fusion and immunohistochemistry evaluation. For spine fusion, 16 sheep were included and randomly divided into autograft and CHA groups, with eight sheep each. PLF was performed at two levels in each sheep: one with BMNCs, and one without. Each sheep served as its own control (Ding et al., 2019; Sorensen et al., 2012).

For immunohistochemistry, eight additional sheep were included for harvesting bone marrow. Bone marrow was aspirated from the iliac crest of each sheep and used for bioreactor activation of the hydroxyapatite/β-tricalciumphosphate-poly(d,l-lactide) (HA/β-TCP-PDLLA, abbreviated as HA/β-TCP).

The BMNCs from one sheep were incubated with two half-cylindrical CHA or HA/β-TCP in a bioreactor chamber house (Figure 1). Subsequently, the harvested samples were prepared for analyses, with one half from each sheep for qualitative histology, and the other half for bone immunohistochemistry (details below) (Sorensen et al., 2012).

### 2.2 *In vivo* posterolateral spine fusion

**Animals**

Skeletally mature (age 3-6 yrs.) female Merino/Gotland wool mixed-bred sheep (Fårebrug Aps, Køge, Denmark) were used in this study. The mean body weight of the sheep was 69.3±8.7 kg in the autograft group, 76.5±5.8 kg in the CHA group and 72.3±3.1 kg in the immunohistochemistry group. No statistical difference in body weight among the groups (P=0.14).

The research protocol was approved by the Danish Animal Experiments and Inspectorates (2008/561-1544) and experiments were performed in accordance with the Danish Animal Research Guidelines and in compliance with the Reporting of *In Vivo* Animal Research Experiments (ARRIVE) Guidelines.

**Bone graft substitutes**

Two types of substitutes were used because of availability at the time of performing experiments.
For implantation in the sheep PLF model, a porous non-stoichiometric bioactive type B CHA (CO$_3^{2-}$ 6-7 wt%) was used. The substitute was manufactured by Fin-Ceramica Faenza Spa (Faenza, Italy) and had a mean porosity of 83±3%, pore diameter of 328±145 µm, and pore interconnections ranging between 50 - 200 µm in diameter, as documented by scanning electron microscopy. Based on a pilot study in the sheep model, the CHA was shaped like a half cylinder (diameter (Ø)=15mm, length (L)=50mm, and volume (V)= 4.418 cm$^3$) (Figure 2). Two half-cylinders were placed against each other to create one cylinder and were fitted to the cylindrical substitute chamber house of the bioreactor (Figures 1 & 3).

For immunohistochemistry, an HA/β-TCP bone graft substitute (Danish Technological Institute, Copenhagen, Denmark) was used. The HA/β-TCP ratio was 70/30 and it was infiltrated with 15% poly(d,l-lactide) (50% d, 50% l) (PLA 50 PHUSIS) to optimize mechanical strength. The HA/β-TCP was shaped like a half-cylinder (diameter Ø=15mm, length (L)=50mm, and volume (V)= 4.418 cm$^3$), so that two half-cylinders were placed against each other in the bioreactor substitute chamber house (Figure 1). The mean porosity of the substitute was 71±2%, with a pore diameter of 300 – 600 µm and pore interconnections ranging between 100 – 250 µm in diameter.

**Bone marrow aspiration and cell isolation**

The sheep were sedated with 1 ml rompun (20 mg xylazin, 1.5 mg methylparahydroxybenzoate, Bayer Animal Health GmbH, Leverkusen, Germany) and anaesthetized with 1.5 - 4.0 mg propofol (Rapinovet, Schering-Plough, Havneholmen, Denmark). Four sites in each iliac crest were identified for aspiration, and local anesthesia was injected in the skin and periost. Subsequently, 4x5 ml of bone marrow was aspirated from the intramedullary cavity using an aspiration needle (11G). The bone marrow aspirate was immediately merged with 4 ml α-MEM (Gibco Minimal Essential Medium, α-Medium, Invitrogen, Denmark) and 1 ml 5000 IE/ml heparine (Heparin, Nycomed, Denmark). The mononuclear cells were isolated by density gradient centrifugation (Histopaque
In the CHA group, mononuclear cells were resuspended in 22 ml α-MEM. In the autograft group, mononuclear cells were resuspended in 10 ml α-MEM media. The total number of BMNCs was counted for each animal.

Bone marrow forming capacity

CFU-f units in the bone marrow aspirate indicated the clonogenicity of the mononuclear cells and an estimate of each sheep bone-forming potential (Braccini et al., 2007). CFU-f units were counted after 10 days of culture plating $10^5$ cells in a 25 mL α-MEM+ in a T-25 flask. In the CHA and immunohistochemistry groups, cells were loaded into the bioreactor (Table 1).

Bioreactor BMNCs seeding and culture

The CHA and HA/β-TCP were prepared and activated in the computer-controlled perfusion bioreactor system following identical procedures (as follows). Prior to BMNC isolation, the bone graft substitutes were press-fitted into the bone chamber and prewashed washed in the substitute chamber house with α-MEM for 4 hours with a replacement of media after the first hour in order to remove loose bone graft material particles and neutralize the pH level (Figure 1). After this procedure, the CHA without BMNCs group was implanted directly into sheep. The CHA with BMNCs group and the HA/β-TCP group received continuous cell seeding and expansion in the bioreactor on the substitute matrix as freshly isolated BMNCs were directly injected into the automated flow perfusion bioreactor’s circulating media. Using this method, the conventional phase of monolayer cell expansion was bypassed, as previously described (Braccini et al., 2007; Braccini et al., 2005) (Figure 1). The seeding phase commenced on day 4, with 22 ml of suspended freshly isolated BMNCs being infused into the bioreactor system. During cell seeding, the media was perfused at a flow rate of 4 ml/min (i.e., superficial velocity of 400 µm/s through the substitute), with a change in the perfusion direction every 30 min. Following cell seeding, the bioreactor system was infused with an additional 40 ml of culture media, which was supplemented with 5 ng/ml
fibroblast growth factor-2 (Recombinant human fibroblast growth factors (FGF) basic- 146- aa, R&D systems, Abingdon, UK), 10nM dexamethasone (Dexamethasone, Sigma-Aldrich, Søborg, Denmark) and 0.1 mM L-ascorbic acid-2-phosphate (L-Ascorbic acid-2-phosphate sesquimagnesium salt hydrate, Sigma-Aldrich, Søborg, Denmark). The flow was then decreased to 1 ml/min (i.e., superficial velocity of 100µm/s) for the remaining culture period, and media was exchanged twice per week.

The perfusion bioreactor was placed inside an incubator (37˚ C, 5% CO₂) throughout the experiment, and O₂ consumption and pH were monitored (Ding, Henriksen, Wendt, & Overgaard, 2016; Sorensen et al., 2012) (Figure 1). The bioreactor is driven by an automatic reverse bar pump, which pumps the media through the substitute chamber in both the seeding and growth periods. The various parts within bioreactors are connected by silicone tubs permeable to oxygen and CO2. Oxygen consumption and pH level are measured by micro-sensors (PreSens, Precision sensing GmbH, Regensburg, Germany) to observe the viability of the cells (Sorensen et al., 2012).

After a total incubation period of 21 days, the substitutes were harvested from the bioreactor chamber house and implanted into the PLF sheep model for the CHA group. The HA/β-TCP bone graft substitutes were subsequently prepared for qualitative histology and immunohistochemistry.

**Surgical procedure**

The sheep were pre-anaesthetized as previously described, and then intravenously anaesthetized with 1 mg/kg propofol and 2 ml of 0.03-mg/ml buprenorphine (Tempgesic, Schering-Plough, Copenhagen, Denmark). Anaesthesia was maintained during surgery with isoflurane 2.5% (Sieste vet. Dameca, Rødovre, Denmark). An incision was made from costa 12 to the os sacrum by sharp dissection. Soft tissues were detached from the facet joints, while spinal and transverse processes were detached by blunt dissection. Preparation of the graft bed was performed via the decortication of the facet joints and transverse processes. All bone chips were left *in situ*. The CHA or autograft
was then implanted into the lateral gutter between transverse processes, respectively, and covered with muscles. In the autograft group, 9 - 10 g of bone chips were harvested from the posterior iliac crest during surgery. If it was not feasible to harvest a sufficient amount of bone from the iliac crest, the additional bone was taken from costa 12 (Sorensen et al., 2012).

In the autograft without BMNC group, bone chips were placed directly at the graft bed, whereas bone chips were mixed in a petri dish with the freshly isolated BMNCs prior to implantation in the autograft with BMNCs group. In the CHA group with/without BMNC, the substitute was taken directly from the bioreactor (as previously described) and then implanted in the sheep. The same consultant spine surgeon (SJ) performed all of the surgeries.

For prophylactic purposes, the sheep were injected postoperatively with 8 ml 250mg/ml ampicillin (Ampivet Vet., Boehringer Ingelheim, Copenhagen, Denmark) daily for 5 days, and with 2 ml buprenorphine 0.03 mg/ml three times daily for 3 days. The sheep were kept for observation in a booth with limited possibility for movement for 3 days post-surgery. Next, they were moved to outdoor farming facilities with shelter and no movement restrictions. After 4.5 months, the sheep were euthanized by an overdose of pentobarbital, and lumbar-sacral segments (L1 - S1) were removed en bloc. The length of the observation period was based on our previous study, which demonstrated that not all sheep are able to fuse by using autograft (Ding et al., 2019; Sorensen et al., 2012).

**Micro-CT scanning**

After harvesting, fusion blocks (approximately 2x2x6 cm) were prepared. Specimens of the autograft group were larger than those of the CHA group and were unable to fit into the scanning tube of micro-CT scanner. The autograft specimens were thus sawed into medial and lateral parts in the horizontal plane. The medial part was scanned and used for further analysis, assuming fusion was uniform throughout the horizontal axis of the fusion block. The specimens were scanned using
a high-resolution micro-CT scanner (vivaCT40, Scanco Medical AG, Brüttisellen, Switzerland) using 70kVp and 84µA. The scanned images had 3D reconstruction cubic voxel sizes of 35x35x35 µm³ (1024x1024x1024 pixels) with 32-bit-grey levels. The scanned images were segmented using previously described methods (Ding, Odgaard, & Hvid, 1999) (Figure 3).

Microarchitectural properties

The microarchitectural parameters were calculated as the mean values for each fusion level (i.e. the mean of left and right sides of the spinous process were considered as one fusion level) and were used for statistical analyses. The region of interest was defined to include new bone between the transverse processes excluding the old bone of the transverse process in the autograft group. The CHA group included remaining substitute.

Thereafter, microarchitectural parameters were calculated. Bone volume fraction (%) was the fraction of newly formed bone in a region of interest. Porosity (%) was the void volume per total volume of the specimen (1 - bone volume fraction). Bone surface to bone volume ratio (mm⁻¹) was the bone surface area to the total volume of the specimen. Trabecular thickness (mm) was calculated from a volume-based local thickness of the trabeculae. Bone material density (mg/cm³) was expressed the degree of mineralization in a volume of interest after thresholding. Bone apparent density is the mass of mineralized tissue (mg) relative to bone volume (mg/cm³). Structure model index (-) is a measure of the shape of the trabecular bone based on a differential analysis of triangulated bone surface (i.e. rods, plates or a combination of these; SMI =0 for ideal parallel plates, 3 for cylindrical rods). Negative values indicate dense, concave structures. The degree of anisotropy (-) is described as the orientation of the trabecular network in terms of preferred orientation (1 indicates isotropy, >1 indicates anisotropy). Connectivity density (mm⁻³) is a measure of multiple connections within the trabecular network in proportion to the total volume (Ding, 2010; Odgaard, 1997). The degree of mineralization was determined as the mass of mineralized tissue.
(mg) relative to its volume (cm³) in a volume of interest (Ding, Cheng, Bollen, Schwarz, & Overgaard, 2010).

**Fusion assessment by micro-CT**

Micro-CT images were 3-D reconstructed and spine fusion was assessed. Fusion was identified and defined when bone trabeculae were presented without interruption between transverse processes. Any uncertainty regarding spine fusion was considered not fused (Ding et al., 2019).

**Histology for qualitative description of bone morphology**

Histology was used as a secondary outcome measure by a qualitative description of the newly formed bone quality (i.e. organization patterns), the morphology of the transition zone between newly formed bone and old bone, any residue of CHA, remodeling activity in general and signs of foreign body reaction and inflammation, etc.

After harvesting, specimens were frozen at -20°C, fixed in 70% ethanol for 16 weeks at room temperature (20 - 22°C) and slowly dehydrated in graded concentrations of ethanol for 7 weeks. Subsequently, they were infiltrated and embedded in methylmethacrylate for 9 weeks. Then, 25 µm-thick undecalcified sections were sawed from the embedded specimens using a specially constructed microtome (Medeja, Instrumentmakerij, Assendelft, the Netherlands). The sections were then stained with toluidine blue and reviewed by microscopy using an Olympus microscope and newCast™ software (Visiopharm, Hoersholm, Denmark) (Figure 4).

### 2.3 Immunohistochemistry evaluation

**Histology for BMNCs 3D distribution assessment**

In order to study cell distribution and to characterize cells within the substitutes, we developed a protocol for processing the decalcified substitutes for histological examination. After bioreactor activation, substitutes adherence with BMNCs was fixed in 4% formaldehyde in phosphate buffer (Bie & Berntsen, Rødovre, Denmark) for 4 days, subsequently rinsed and then stored in phosphate buffer.
buffer (Amplicon bioreagents & molecular diagnostics, Skovlunde, Denmark) for another 4 days. Next, they were infiltrated with bovine serum albumin 15% (Sigma-Aldrich, Brøndby, Denmark) in a vacuum chamber and exposed to glutaraldehyde 15% vapours (Merck, Darmstadt, Germany) until the albumin was coagulated.

Thereafter, the substitutes were decalcified for 3 hours (KOS Microwave HistoSTATION, Milestone, CA, USA), dehydrated in graded alcohols and paraffin embedded (Histolab products AB, Gothenburg, Sweden). Then, 4 µm sections were cut using a microtome (Leica microsystems, GmBh, Wetzlar, Germany) for hematoxylin and eosin (HE) staining and immunohistochemistry. Sections from zones representing the exterior (equally exposed to flow gradient in both directions) of the substitutes were selected (Figure 5). The distribution and characterization of BMNCs within substitutes were described by qualitative histology also using an Olympus microscope and newCastTM software.

**Immunohistochemistry**

Immunolocalisation of Ki67 (rabbit monoclonal Ab30-9, Confirm, Ventana Medical System), vimentin (mouse monoclonal Ab V9, Confirm, Ventana Medical System), osteonectin (mouse monoclonal Ab 15G12, NovoCastra) and osteopontin (mouse monoclonal ab OP3N, Novo Castra) were performed on 3µm-thick sections of the bioreactors (proceeded as described above) in order to illustrate the proliferation potential and differentiation of the BMNCs (Rhys, 2002) (Table 4, Figure 6). Staining was performed on a Ventana, Benchmark platform (Roche). All stainings included controls and Ki67 stains served as a marker of mitosis (Schonk DM, 1989), whereas vimentin confirmed the cells originating from the mesenchymal cell line (Eriksson JE, 2009). Osteonectin and osteopontin were expressed by cells in proliferation and differentiation (Yang, Davies, Archer, & Richards, 2003). Therefore, these immune stains confirmed BMNCs being part of the
regenerative process (Table 4). The manufacturer provided all immune stains with a positive stain enclosed for comparison.

**Statistical analysis**

Assuming that un-instrumented PLF fusion frequency in humans is approximately 80% (Steinmann & Herkowitz, 1992), the minimum number of animals required in each group was 7.12, provided $\alpha=0.05$ and $p=0.8$. Fusion rastes were tested by Fischer’s exact test. Microarchitectural data were presented as mean ± SD. The paired effects of ±BMNCs on the microarchitectural parameters in the CHA and the autograft groups, were first evaluated by the Wilcoxon’s matched-pairs signed-rank test. No significant differences were found in the autograft group, hence the two groups ±BMNCs were merged and named “autograft group” for further analysis. Differences between the groups were assessed by one-way ANOVA. Multiple post hoc comparisons were adjusted using Tamhane’s test. Changes in porosity were compared from an initial value to the CHA group with and without bioreactor activation using the Wilcoxon’s matched-pairs signed-rank test. Results were considered significant when P values were less than 0.05. The statistical analyses were performed using IBM SPSS statistics version 25, Chicago, USA.

3 | RESULTS

3.1 Exclusion of animals and bioreactor-activated substitutes

In the autograft group, three sheep were euthanized before the end of the intended observation time due to 1) a paralytic hind leg, 2) paralytic rumen, and 3) a shoulder injury acquired during surgery as a result of positioning, respectively. One additional sheep was included in the autograft group to compensate for the lost sheep. As such, the number of sheep in the autograft group for the final analysis was reduced from eight to six. In the CHA group no CFU was found in bone marrow aspirates in two sheep; therefore, a deficient bone-forming capacity was anticipated in these animals.
and they were excluded. Thus, there was also a reduction of sheep from eight to six in CHA group in the final analysis.

Due to technical problems in the laboratory concerning the CO₂ supply for the incubators during the incubation of HA/β-TCP, four bioreactor runs from four sheep were excluded, leaving only two HA/β-TCPs per sheep for further qualitative analysis. In total, eight HA/β-TCPs from four sheep were used for histology and immunohistochemistry.

3.2 Fusion assessment by micro-CT images

The numbers of BMNCs used in the autograft and the CHA groups are summarized in Table 1a. Notably, there was an uneven number of BMNCs prior to transplantation. The number of BMNCs loaded into each bioreactor was presented and however the higher number of BMNCs did not always result in higher CFUs. No dose-respond effect was observed reflecting a large biological variation (Table 1b). The fusion rate in the CHA group was 5/6 (83.3%) when activated in the bioreactor with BMNCs, and 4/6 (66.7%) in the absence of BMNC. No statistical difference between these two groups (P=1.00) was observed. The autograft group had a healing rate of 5/6, irrespective of the presence of BMNCs. One sheep did not exhibit spine fusion on both levels, irrespective of BMNCs (Table 2).

3.3 Microarchitectural properties of the fusion mass

Bone volume fraction was significantly greater in the autograft group compared to the CHA±BMNCs groups (Table 3). Porosity decreased significantly from 83.3% before implantation to 45.4% and 46.9% in the CHA±BMNCs groups, respectively (P=0.03, Wilcoxon's matched-pairs signed-ranks test). No difference was observed between the autograft ±BMNCs groups. The bone surface-to-volume ratio was significantly lower in the autograft group compared to the CHA with BMNCs. Trabecular thickness was significantly greater in the autograft group compared to the CHA without BMNCs group. Structure model index was significantly lower in the autograft group...
compared to CHA±BMNCs groups, and the degree of anisotropy was significantly higher in the autograft group compared to CHA with BMNCs. Connectivity density was significantly lower in the autograft group compared to CHA with BMNCs. Bone material density was significantly lower in the autograft group compared to CHA±BMNCs groups (Table 3).

**Histological qualitative assessment of bone morphology**

Histological sections of the fusion mass in the autograft and CHA groups are presented in Figure 4. In both groups, new bone was remodelled into lamellae, suggesting intramembranous bone formation (a). In the autograft group, new bone osseointegrated well with pre-existing bone upon the transverse processes (a+b) and showed signs of active remodelling with active osteons depositing osteoid (a+b). The surface of the BGS with cells migrates towards the core (b), and a cluster of cells not viable in a densely populated cluster (c). In the CHA group, substitute remnants were found in all fusion blocks. Newly-formed bone was osseointegrating well with CHA remnants and pre-existing bone (d+e). Bone formation was initiated either from the pores of the CHA (f) or from the surface. Osteoid deposition occurred within the luminae, and very early organization of the woven bone (g) was observed. The pores were filled with fibrous tissue in cases of fusion blocks without BMNCs (h+i) (Figure 4).

**Histological assessment of BMNCs distribution and phenotype**

Histological sections of the seeded HA/β-TCP (Figure 5) displayed the BMNC distribution pattern. The presence of albumin in all pores of the HA/β-TCP suggested that all portions of these scaffolds were accessible for perfusion (Figure 5A). All sections showed infiltration of the HA/β-TCP by BMNCs. However, more BMNCs were present near the surfaces of the HA/β-TCPs (Figure 5B-D); in particular, a layer of vital cells covered much of the outer surface of the scaffold half-cylinders. Fewer or no cells were observed towards the HA/β-TCP core (Figure 5E and F). BMNCs could be found densely populating pores (Figures 5D and 6C) or lining the surfaces of larger pore surfaces.
(Figures 5B, D and E and 6 B). In the densely populated pores, degeneration of cells in the central parts was observed (Figure 5B), though degeneration could also be detected in deeper portions of cell clusters where cells opposing the free pore lumen appeared vital (Figure 5B). Moreover, pores with degenerating cells were found, while those in the neighbouring pore appeared vital (Figure 5C).

Immunohistochemistry (Figure 6) showed staining for vimentin in all cells in the HA/β-TCPs, thus demonstrating their mesenchymal origin. Ki67 staining was found mainly in cells adjacent to the pore surface, while osteopontin- and osteonectin-expressing cells were found within cells in the cell clusters inside the pores. Osteopontin, osteonectin and Ki67 stained weaker than the control tissues, which was likely due to the exposure of the material to glutaraldehyde. The Ki67 expression demonstrated that BMNCs in the seeded HA/β-TCP were able to proliferate and that osteonectin and osteopontin reactivity affected their ability to differentiate (Table 4).

4 | DISCUSSION

This pre-clinical study evaluated the efficacy of a large-sized CHA bone graft substitute with or without isolated BMNCs in a highly challenging large animal PLF model without instrumentation. Our results demonstrated that the presence of BMNCs enhanced fusion rate by 66.7% to 83.3% in the CHA group, which was the same fusion rate in the autograft group, irrespective of the presence of BMNCs. A significant decrease in porosity (p=0.001) in the CHA group suggested the deposition of mineralized bone. To reveal the bioreactor effects on cell seeding on a substitute in more detail, the distribution, proliferation and differentiation of BMNCs were assessed using a HA/βTCP substitute subjected to bioreactor activation with BMNCs. The characterization of BMNCs by immunohistochemistry confirmed the ability of the bioreactor to incubate large-sized substitutes with viable BMNCs with the potential for proliferation and differentiation. While the homogenous
3D distribution of the cells within large substitute was not observed, but cells were found to migrate towards substitute core. To the best of our knowledge, the testing of large-sized bioreactor-activated substitutes in a large animal PLF model, has only been performed by our group (Sorensen et al., 2012). In light of the results of the present study, the authors suggest a better fusion capacity in the bioreactor-generated bone constructs compared to controls without cells.

**Limitations**

The sample size was reduced from eight to six in both the CHA and the autograft groups, which demonstrates that the model is challenging. In the HA/βTCP group, the number was reduced from eight to four, leading to an observational investigation only. Furthermore, two different types of substitutes were used in two studies due to the availability. Although they are similar in many ways, we cannot neglect or delimitate potential differences between them. We believe that it is more valuable to combine these two datasets. As such, the interpretation of the results should be performed with caution; however, this should not comprimize the clinical significance of the study. Our results demonstrate a successful enhancement of BMNCs on fusion rate in the CHA group comparable to the autograft group, and the characterization of BMNCs within CHA.

**Sheep PLF model**

The sheep PLF model without instrumentation is considered a highly challenging model for studying spine fusion and bone regeneration that carries translational potential. For successful fusions, the PLF model relies on biological factors in substitute rather than as mechanical support. The model is characterized by tensile forces at the site of bone healing. Therefore, osteogenic and osteoinductive factors are of great importance relative to mechanical support, which increases the requirements of the employed substitute (Boatright, 2005). Moreover, multilevel fusions were performed in each animal in this study, which was associated with lower fusion rates (Steinmann & Herkowitz, 1992).
**Fusion efficacy**

In our pilot studies, we expected and found no fusions in the substitute without BMNCs group. The CHA group had 5/6 cases fused when activated in the bioreactor with BMNCs, and 4/6 cases fused without BMNCs, which resembles the fusion rates of autograft reported in humans and is equal to that of the autograft group (Ludwig, Kowalski, & Boden, 2000). Our results demonstrated the presence of BMNCs enhancing the fusion rate from 66.7% to 83.3% in the CHA group, which was the same fusion rate in the autograft group, irrespective of the presence of BMNCs. Micro-CT images clearly revealed CHA was not fully reabsorbed. Therefore, it can be anticipated that additional remodelling cycles would improve bone mass quality.

**Microarchitecture and bone-forming capacity**

Significant differences between the groups regarding the microarchitectural properties of the fusion masses were observed. Bone volume fraction and trabecular thickness were significantly greater, while the bone surface-to-volume ratio was lower in the autograft group compared to the CHA group with and without BMNCs. This indicates the presence of more newly-formed bone in the autograft group relative to the CHA group, due to a more rapid remodelling in autograft than in synthetic bone substitutes (Schimandle, 1997). Porosity decreased significantly in the CHA group with and without BMNCs after implantation, thus indicating new bone deposition. Kruyt et al. (Kruyt.M.C., 2006) found a similar positive effect of BMNCs on bone formation, mostly pronounced in non-bony contact areas in a goat transverse process model comparable to the PLF model used in this study.

In all three groups, a concave plate-like cancellous bone structure were observed via negative SMI values. The autograft group had a greater degree of anisotropy than the CHA group, thus suggesting the relatively better orientation of the trabecular network to support mechanical loading and a more mature fusion mass. As expected, the autograft had significantly lower bone material density
compared to the CHA group with and without BMNCs, i.e. the new bone in the autograft group was less dense compared to the mixture of substitute remnants plus new bone in the CHA group. Taken together, fusion blocks in the autograft group revealed more bone with thicker trabeculae and better trabecular orientation, but less connection compared to the CHA group, while both groups had acceptable fusion mass, bone remodelling and fusion rate.

**Material properties**

Hydroxyapatite (HA) is an attractive material for bone tissue engineering due to its high bioactivity and ability to osseointegrate (LeGeros, 2002). While HA has been widely tested in various animal and fracture healing models, in critical-sized defects, it is inadequate as a bone filler and to promote solid bone healing without a supplementary biologic factor to induce osteoinductivity and/or osteogenecity (Cancedda et al., 2007).

Compared to HA, CHA is considered more biomimetic due to its chemical composition being closer to that of natural bone. Non-stoichiometry and low crystallinity make this compound more soluble and thus more bioactive. Halstenberg et al. (S Halstenberg, 2006) found a positive effect of the carbonate ion on osteoblastic cell differentiation and suggested improving vascularization potential. In turn, this implies more rapid osseointegration and improved biocompatibility (Brydone, Meek, & Maclaine, 2010).

**Influence of bioreactor activation with BMNCs**

The major concerns when seeding large-sized bone graft substitutes with BMNCs included: 1) achieving a 3D uniform distribution of cells within the substitute; 2) obtaining a sufficient number of cells to promote bone formation; 3) avoiding hypoxia inside substitute core; and 4) expansion of BMNCs before seeding on to the substitutes, resulting in low seeding efficiency (Izadpanah et al., 2008). In the present study, these challenges were not studied in detail; however, we used flow perfusion bioreactors that have been proposed to overcome some of these obstacles. It is widely
considered the preferable bioreactor for bone tissue engineering due to its ability to transport media to and from the substitute core (Meinel et al., 2004), while the perfusion should ensure an uniform distribution of BMNCs within the substitute (Burdick & Vunjak-Novakovic, 2009), and the deposition of mineralized matrix (Braccini et al., 2005; Janssen, Oostra, Oorschot, & van Blitterswijk, 2006). It is suggested to implement more advanced in situ measurement devices for allowing for true real-time, on-line metabolite monitoring and cellularity estimation (Simmons, Williams, Degoix, & Sikavitsas, 2017).

In this study, we could not reproduce the bioreactor facilitated with a monogenous 3D distribution of BMNCs within large-sized substitutes. The number of cells was much larger at the top and bottom ends of the substitutes i.e. in the zones directly and equally exposed to the media flow. This may be explained by the effect of flow gradually decreasing towards the core of the substitute, with a major impact on the distribution of cells. There were no signs of cell attachment in the substitute interior in opposition to the surface and superficial areas where viable cells were found. The cells adhered in two patterns: predominantly by lining the surface of the substitute in a thin layer, and secondly by occupying the pores in the substitute matrix in larger numbers. Examples of non-viable cells in very densely populated pores were found, suggesting that incubation of the bioreactor with very large numbers of cells is not favorable for cell survival.

The distribution of cells appeared to reflect the perfusion regime, with more cells in superficial pores and along the outer surface. However, some cells also reached the central part of the HA/β-TCPs. The presence of densely populated pores combined with immunohistochemical demonstration of Ki67 also indicates that a local expansion of cells had occurred. At the time of analysis, Ki67-positive cells were primarily found on the substitute surface and adjacent to substitute pore exteriors. Cells in the interior of the substitute pores and in small cell clusters were more differentiated and less prone to proliferation reflected by the presence of osteopontin and
osteonectin positive cells (Hejbol, Sellathurai, Nair, & Schroder, 2017) (Table 4). Differentiation towards the osteoblastic cell line seemed more attractive for BMNCs that adhered centrally in the substitute matrix, which could be explained by the exposition to different perfusion velocities compared to BMNCs in more superficial compartments. In this connection, it can be stated that a reduction in oxygen and nutrients has been described, which induces differentiation (Cheng, Markenscoff, & Zygourakis, 2009; Hejbol et al., 2017). The presence of many vital cells in the superficial part of the scaffolds could be advantageous in relation to in vivo integration. Moreover, the presence of degenerated cells in the scaffolds likely reflects an imbalance between available oxygen and nutrients and the number of cells. This could be the result of the extensive proliferation of cells in a pore, which resulting in it being filled and thus reusing the perfusion or that some pores are less perfused than others and thus capable of supporting fewer cells. To reduce cell death, one might consider reducing the in vitro incubation time.

This leads to the question of whether the 3D uniform distribution of BMNCs within a given substitute is crucial for bone formation and bone healing. It is probable that the presence of viable, proliferating and bone-forming cells (though not penetrating to the substitute core) is sufficient to promote bone formation and bone healing in cooperation with host osteogenic cells. In a recent study, Zhou et al. (Zhou, Fan, Prasadam, Crawford, & Xiao, 2012) noted that osteogenetic differentiated human mesenchymal stem cells were able to recruit host cells in a severe combined immune-deficient mouse (SCID) model. A migration assay analysis revealed host mouse bone-forming cells being predominantly involved in new bone formation compared to donor cells. Therefore, the authors would like to draw further attention to the interaction between donor and host cells in cell therapy strategies.

In general, there are several key elements needed to run a successful study in such a very changing bioreactor system: 1) follow description of methodology using a well-designed computer-controlled
perfusion bioreactor system; 2) continuously monitor cell survival via oxygen consumption and pH level during the culture period; 3) apply a constant flow rate of 4 ml/min and change medium in due time; 4) seed adequate amount of cells, e.g., 3-6x10^7; and 5) place the bioreactor inside an incubator (37˚ C, 5% CO₂) throughout the experiment.

5 | CONCLUSION
Overall, the findings of this study suggest that the bioreactor-activated substitute presented fusion rates and microarchitecture comparable to autograft. Fusion blocks in the autograft group generated more bone with thicker trabeculae and better orientation, but less connection compared to the CHA group, while both groups had acceptable fusion mass, bone remodelling and fusion rates. Based on immunohistochemistry, the CHA group loaded with BMNCs demonstrated osteoconductive and osteogenetic properties despite the limited uniformity in cell distribution. It is feasible that new functional bone regeneration could be achieved by the combination of BMNCs, biomaterials and bioreactors. However, it requires further evaluation regarding the efficacy and improved distribution of BMNCs within scaffold in this model.

ACKNOWLEDGEMENT
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Gitte Reinberg for technical assistance, and the staff at the Biomedicine Laboratory, University of Southern Denmark for handling experiment animals.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION
M.D., K.E.K., S.O., and H.D.S. designed the study; K.E.K., wrote the manuscript; M.D. wrote partial manuscript and thoroughly revised the manuscript; S.J and K.E.K.. performed spine surgery; K.E.K., M.D., and H.D.S. performed experiments and collected data; R.M. produced and provided CHA; D.W. and I.M. provided technical support for the bioreactor. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS

FIGURE 1 Diagram of the bioreactor from the AUTOBONE reports. Specially designed software controlled the culture period sequences. The media was perfused through the substitute chamber
house in a system of tubing connected to reservoirs and bottles of culture and waste medium. Flow direction and velocity was controlled by a pump and valves. O₂ and pH levels were monitored by sensors.

**FIGURE 2**  Left: The half-cylinder CHA. Right: The differences in porous microarchitecture and size between the CHA and pure HA are illustrated by scanning electron microscopy.

**FIGURE 3** Illustration of bioreactor incubated large cylindrical substitute. Bioreactor chamber housing two half cylinders during the culture period (A). BMSC seeding of half cylinders throughout the culture period (B & C). Transplantation of cultured cylinder onto posterolateral side between two transverse processes (D). 3-D reconstruction of spine fusion block (E) and thin cross section layer (F) of micro-CT images.

**FIGURE 4** Histological sections of fusion blocks. **Zone 1:** (A) x40: viable cells in clusters and lamellar bone; (B) x20: the surface of the CHA with cells migrating towards the core; and (C) x20: a cluster of cells not viable in a densely populated cluster. **Zone 2:** (D+E) x10: core and periphery of fusion block. **Zone 3:** (F) x10: core CHA of the convex side. Bone formation was initiated either from the pores of CHA (F) or from the surface. Osteoid deposition within the laminae, and very early organization of the woven bone (G) x40. The pores were filled with fibrous tissue in cases of fusion blocks without BMSCs (H+I) x10.

**FIGURE 5** Histological H+E stained sections of the bioreactor-activated HA/β-TCP. The cross-section of a decalcified half-cylinder (A) shows all pores filled with albumin (pink staining). Dark, granulated and empty areas represent the residue of the scaffold. In the periphery of the half-cylinders (B, C and D), many pores contained cells. Cells were found on most of the outer surface of the half-cylinders (D arrows). In superficial pores, both vital (B and C, white arrows) and degenerating cells with condensed, dark nuclei (B and C, black arrows) could be found. In the central part of the half-cylinders, pores with cells (E) were present; however, large areas consisted
of empty pores (F). In the central pores, both vital and degenerating cells were also present (white and black arrows, respectively).

**FIGURE 6** Immunohistochemistry of HA/β-TCP incubated BMNCs in the flow perfusion bioreactor. Sections stained for vimentin demonstrated the presence of BMNCs on the outer surfaces (A) and in the pores of the scaffold (B) (arrows). Ki67 positive nuclei in cells at surfaces of the pores and in small cell clusters (C and D, arrows), while osteopontin (E, arrow) and osteonectin (F, arrow) stained cytoplasm in cells in the cell clusters. Circle in C highlights area with degenerated nuclear material.

**REFERENCES**


10.1016/j.biomaterials.2005.07.044


TABLE 1a  Number of BMNCs used in the autograft and CHA groups.

<table>
<thead>
<tr>
<th>Autograft BMNCs x 10^7</th>
<th>CHA BMNCs x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.23</td>
<td>25.2</td>
</tr>
<tr>
<td>5.87</td>
<td>5.0</td>
</tr>
<tr>
<td>4.95</td>
<td>76</td>
</tr>
<tr>
<td>5.74</td>
<td>48</td>
</tr>
<tr>
<td>3.99</td>
<td>5.1</td>
</tr>
<tr>
<td>3.59</td>
<td>4.1</td>
</tr>
</tbody>
</table>

1b Number of BMNCs loaded into bioreactor & outcount CFU

<table>
<thead>
<tr>
<th>Number of BMNCs loaded into the bioreactor</th>
<th>CFU/100,000 BMNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.68x10^7</td>
</tr>
<tr>
<td>2</td>
<td>5.82x10^7</td>
</tr>
<tr>
<td>3</td>
<td>4.34x10^7</td>
</tr>
<tr>
<td>4</td>
<td>2.68x10^7</td>
</tr>
</tbody>
</table>
**TABLE 2** Number of fused and non-fused explants assessed by micro-CT.

<table>
<thead>
<tr>
<th></th>
<th>no fusion</th>
<th>fusion</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>without BMSC bioreactor activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autograft</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>CHA</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td><strong>with BMSC bioreactor activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autograft</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>CHA</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5 (20.8%)</td>
<td>19 (79.2%)</td>
<td>24 (100%)</td>
</tr>
</tbody>
</table>
TABLE 3  3-D microarchitectural properties of sheep spine fusion blocks by groups.
G1: Autograft +/- BMSCs, G2: CHA - BMSCs. G3: CHA+BMSCs. Means ± SD. P-values obtained by ANOVA and Tamhane’s post hoc test. Porosity was compared to the prevalue of 83.3% and tested by Wilcoxon’s signed rank test. P < 0.05 are considered significant.

<table>
<thead>
<tr>
<th></th>
<th>Bone volume fraction (%)</th>
<th>Porosity (%)</th>
<th>Bone surface density (mm⁻¹)</th>
<th>Bone surface to volume ratio (mm⁻¹)</th>
<th>Trabecular thickness (mm)</th>
<th>Structure model index (-)</th>
<th>Degree of anisotropy (-)</th>
<th>Connectivity density (mm⁻³)</th>
<th>Trabecular number (mm⁻¹)</th>
<th>Trabecular separation (mm)</th>
<th>Bone material density (mg/cm³)</th>
<th>Bone apparent density (mg/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G1</strong></td>
<td>66.9±5.5</td>
<td>33.1 ±7.2</td>
<td>3.5 ±0.6</td>
<td>5.0 ±0.5</td>
<td>428±72</td>
<td>-5.1 ±0.8</td>
<td>1.3 ±1.5</td>
<td>4.9±1.7</td>
<td>2.2±0.1</td>
<td>0.5±0.03</td>
<td>678.7 ±40.4</td>
<td>454.4±29</td>
</tr>
<tr>
<td><strong>G2</strong></td>
<td>53.1±4.0</td>
<td>46.9 ±4.0</td>
<td>3.4 ±0.7</td>
<td>6.9 ±1.4</td>
<td>305±35</td>
<td>-2.7 ±1.1</td>
<td>1.1 ±0.02</td>
<td>9.8 ±3.7</td>
<td>2.3±0.07</td>
<td>0.5±0.05</td>
<td>854.6±29</td>
<td>462.6 ±50.5</td>
</tr>
<tr>
<td><strong>G3</strong></td>
<td>54.6±4.7</td>
<td>45.4 ±4.7</td>
<td>4.6 ±1.2</td>
<td>7.5±1.0</td>
<td>333±66</td>
<td>-2.3 ±0.5</td>
<td>1.08 ±0.03</td>
<td>11.0±2.8</td>
<td>2.4±0.2</td>
<td>0.4±0.03</td>
<td>857.0 ±29</td>
<td>449.2±25.3</td>
</tr>
<tr>
<td><strong>ANOVA P-value</strong></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G1&gt;G2,G3</td>
<td>P&lt;0.001</td>
<td>P=0.6</td>
<td>P=0.002</td>
<td>P=0.002</td>
<td>P&lt;0.002</td>
<td>P=0.002</td>
<td>P= 0.005</td>
<td>P=0.005</td>
<td>P=0.05</td>
<td>P=0.21</td>
<td>P&lt;0.001</td>
<td>P=0.8</td>
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<tr>
<td><strong>Wilcoxon P-value</strong></td>
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</table>
TABLE 4 At different maturation stages, different proteins are expressed by the BMNCs. Detecting the presence of these proteins by immunohistochemistry enables the cells in the bioreactor activated bone graft substitute to be characterized as mitotic, proliferative or differentiated.

<table>
<thead>
<tr>
<th>Protein</th>
<th>G0</th>
<th>Fibroblast</th>
<th>Pre osteoblast</th>
<th>Osteoblast</th>
<th>Osteoid/Osteocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki 67</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+ (+)</td>
<td>+</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: Modified from (Schonk, Kuijpers et al. 1989, Rhys 2002, Eriksson, Dechat et al. 2009)

Zone 1 magnification: viable cells and lamellar bone (a) x 40, surface of substitute (b) x 20 and cluster of cells (c) x 20

Zone 2 magnification: core (d) x 10 and periphery (e) x 10 of substitute. Zone 3: core of substitute from convex side (f) x 10

Magnification: Pore of substitute (g) x 40, pores filled with fibrous tissue (h & i)

(old bone) new bone

scabloid remnant

fibrous tissue

necrotic deposition