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ORIGINAL ARTICLE

Effects of a perfusion bioreactor activated novel bone substitute in spine fusion in sheep

Jesper Roed Sørensen · Kariatta Ester Koroma · Ming Ding · David Wendt · Stig Jespersen · Maria Vinther Juhl · Naseem Theilgaard · Ivan Martin · Søren Overgaard

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Abstract
Purpose To evaluate the effect of a large perfusion-bioreactor cell-activated bone substitute, on a two-level large posterolateral spine fusion sheep model.
Methods A 50 mm long porous biphasic-calcium–phosphate bone substitute reinforced with poly(D,L-lactide) and, activated with bone marrow derived mononuclear-cells (BMNC) was used. Eighteen sheep were divided into two groups and one group ($n = 9$) had BMNC-activated bone substitutes and cell-free substitutes implanted. The second group ($n = 9$) had autograft supplemented with BMNC and regular autograft implanted. The implant material was alternated between spine level L2–L3 and L4–L5 in both groups. MicroCT was used to compare the spine fusion efficacy and bone structure of the two groups as well as the implanted bone substitutes and non-implanted substitutes.
Results After 4½ months six sheep survived in both groups and we found five spine levels were fused when using activated bone substitute compared to three levels with cell-free bone substitute ($p = 0.25$). Five sheep fused at both levels in the autograft group. A significant increased bone density ($p < 0.05$) and anisotropy ($p < 0.05$) was found in the group of activated bone substitutes compared to cell-free bone substitute and no difference existed on the other parameters. The implanted bone substitutes had a significant higher bone density and trabecular thickness than non-implanted bone substitutes, thus indicating that the PLA reinforced BCP had osteoconductive properties ($p < 0.05$). No effect of the supplemented BMNC to autograft was observed. The autograft group had a significant higher bone density, trabecular thickness and degree of anisotropy than the implanted bone substitutes ($p < 0.05$), but a lower connectivity density existed ($p < 0.05$). This indicates that though the activated substitute might have a similar fusion efficacy to autograft, the fusion bridge is not of equal substance.
Conclusion We found that bioreactor-generated cell-based bone substitutes seemed superior in fusion ability when compared to cell-free bone substitute and comparable to autograft in fusion ability, but not in bone structure. This combined with the favorable biocompatible abilities and strength comparable to human cancellous bone indicates that it might be a suitable bone substitute in spine fusion procedures.

Keywords Bone graft substitute · Poly(D,L-lactide) enhanced hydroxyappatite/β-tricalciumphosphate · Perfusion bioreactor · Posterolateral spine fusion · Microarchitecture

J. R. Sørensen and K. E. Koroma contributed equally to this paper.

J. R. Sørensen (✉) · K. E. Koroma · M. Ding · S. Jespersen · S. Overgaard
Department of Orthopaedics and Traumatology, Odense University Hospital, Institute of Clinical Research, University of Southern Denmark, J. B. Winsløwsvej 15 st.th., 5000 Odense C, Denmark
e-mail: j.roed.sorensen@gmail.com

D. Wendt · I. Martin
Departments of Surgery and of Biomedicine, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland

M. V. Juhl · N. Theilgaard
Centre for Plastic Technology, Danish Technological Institute, Gregersensvej 3, 2630 Taastrup, Denmark

Introduction

The posterolateral intertransverse spine fusion procedure is used to treat a wide range of diseases such as critically

lumbar scoliosis, spondylolistesis, spondylosis, degenerative disorders, and back instability. The gold standard graft material for the procedure is autograft characterized by having osteogenic, osteoconductive, and osteoinductive properties [1]. The use of autograft is associated with complications such as extensive callus formation, and chronic donor site pain. In addition abscess formation, chronic pain on walking, nerve damage, and other minor problems can occur. Moreover, if revision surgery is necessary it is not possible to harvest bone from the same site and in many patients it is not possible to harvest the necessary graft amount [2–4].

There exist several alternatives to autograft, but due to various complications with the alternatives autograft is still the gold standard. Biphasic-calcium–phosphates (BCP), which often are used as an alternative to autograft only have osteoconductive properties and additionally are inconveniently brittle wherefore some enhancement is needed (reviewed in [5–7]). The brittleness can be overcome by polymer coatings such as polylactic acid (PLA) used in this study, which enhance the mechanical strength of the substitute [8, 9].

In order to make the BCP more bioactive, a novel perfusion bioreactor-based approach is applied for homogenous seeding, proliferation and differentiation of bone marrow-derived mononuclear cells (BMNC) in the bone substitute. The perfusion bioreactor has advantages in nutrient delivery and waste removal, compared to other bioreactor designs [10–13].

The purpose of this study was to investigate the fusion efficacy and bone structure of a clinical relevant sized PLA reinforced bone substitute activated with BMNC in a perfusion bioreactor and compare it to autograft in a posterolateral spine fusion animal model. We hypothesized bioreactor activated bone substitute and autograft to have similar fusion rates, and that the bone density in bioreactor activated substitutes was increased compared to non-activated cell-free bone substitutes.

Materials and methods

Animals

Eighteen skeletal mature ewes of a Merino/Gotland mixed breed (Fårebrug Aps, Køge, Denmark) with a mean weight of 69 kg (range 55–82 kg) were included in this study. The experimental protocol was in accordance with the Danish animal research guidelines, approved by the Danish Animal Experiments Inspectorate and followed "Principles of laboratory animal care" (NIH publication No. 86–23, revised 85).

Study design

A paired design was used to evaluate the effects of the bioreactor activated substitute. The 18 sheep were divided into two groups and each sheep had two different fusion materials implanted. The bone substitute group ($n = 9$) had activated and non-activated bone substitute implanted while the autograft group ($n = 9$) had regular autograft and BMNC supplemented autograft implanted. The implant locations were alternated among the two spine levels (L2–L3 and L4–L5) by randomization (Fig. 1). Moreover, pure non-implanted bone substitute was included as control to implanted substitutes in the assessment of microarchitecture.

Materials

The BCP powder was provided by PHUSIS® (Saint-Ismier, France) and had stoichiometric hydroxyappatite/β-tricalciumphosphate (HA/β-TCP) composition of 70 % HA and 30 % β-TCP. The substitute including reinforcement was produced by the Danish Technological Institute (Copenhagen, Denmark). The substitute was infiltrated with 15 % poly(D,L-lactide) (D-PLA 50 %, L-PLA 50 %) provided by PHUSIS® (Saint Ismier, France) which gave the scaffold a significant higher stress value around 4 MPa ($p = 0.001$) and stiffness around 250 MPa ($p = 0.01$) compared to pure HA/β-TCP ($p = 0.001$) making the material comparable to human cancellous bone in mechanic properties [14–17]. The substitute was formed as a half cylinder with a length of 50 mm and a diameter of 15 mm and a ratio of 7.5 mm. The total 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 (Fig. 2).

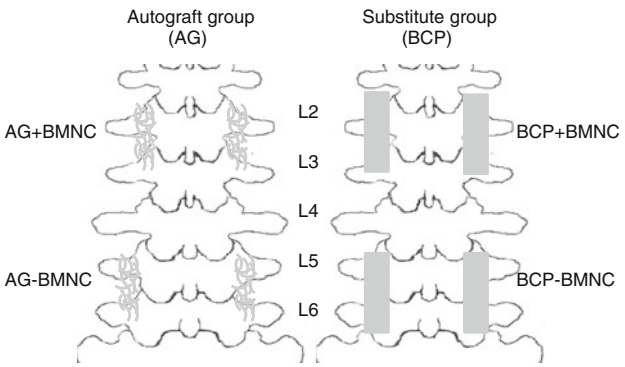

Fig. 1 Study design. A group had randomly autograft supplemented with bone marrow derived mononuclear cells (BMNC) at one level and regular autograft implanted at another level. The other group had randomly bioreactor activated bone substitute with BMNC (BCP + BMNC) and non-activated substitute without BMNC (BCP-BMNC) implanted. Spine fusion was performed at level L2–L3 or L5–L6

Harvest of bone marrow aspirate and cell isolation

BMNC for the autograft group and BMNC for the substitute group were harvested and isolated in an approximately 3½ h long procedure. The sheep were premedicated with 1 ml of a 20 mg xylazin and 1.5 mg methylparahydroxybenzoate solution (Rompun Vet., Bayer, Germany) and anesthetized with 15–40 mL propofol (Rapinovet, Schering-Plough, Copenhagen, Denmark).

Local anaesthesia (Lidokain, Amgros, Copenhagen, Denmark) was applied at two areas on each posterior iliac crest, and two 5 mm incisions was made at each iliac crest followed by 5 ml bone marrow aspiration from each incision site in a 20 ml syringe (Plasticpak, BD Medical, Frankling Lakes, USA) producing a total 20 ml bone marrow aspirate from each sheep. Each aspirate was immediately after aspiration transferred to a 50 mL falcon tube containing 4 ml α-MEM (Gibco Minimal-Essential-Medium, α-Medium, Invitrogen™, Taastrup, Denmark) and 1 ml 5,000 IE/ml heparine (Heparin, Nycomed, Copenagen, Denmark). For both groups the BMNC in the combined aspirate from each individual were isolated using density-gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). The BMNC was resuspended in 10 ml α-MEM for the autograft group and for the substitute group resuspended in a 22 ml media solution (here forth referred to as AM) containing α-MEM, 10 % Foetal-Calf-Serum (Foetal-Calf-Serum, Fischer-Scientific, Copenhagen, Denmark), buffer (HEPES-buffer solution, Invitrogen™, Taastrup, Denmark), Sodium-pyruvate (MEM-Sodium-Pyrovate, Invitrogen™, Taastrup, Denmark), and a penicillin–streptomycine-solution (L-glutamine-Penicillin–Streptomycin, Invitrogen™, Taastrup, Denmark) for the substitute group.

The cells were counted before injection in the perfusion bioreactor or mixing with autograft. The median cell number in the autograft group was 4.5×10^7 and in the bioreactor group 7.3×10^7.

Cell seeding and culture

Freshly isolated BMNC were directly seeded and expanded within the pores of the bone substitute, bypassing the conventional phase of monolayer cell expansion using the perfusion bioreactor system shown in Fig. 3 [11, 18–20].

Prior to BMNC isolation, the bone substitutes were press-fitted into the bone chamber and prewashed with α-MEM for 4 h with a change of media after the first hour. The 22 ml AM solution with BMNC obtained in the harvest procedure was added to the bioreactor and the seeding period set to 4 days with a perfusion flow of 4 ml/min (i.e., superficial volume flow rate of 400 μm/s through the scaffold). The media was shifted between the vertical reservoirs every 30 min. After the seeding period, 40 ml of AM solution was supplemented with 5 ng/ml fibroblast growth factor-2 (recombinant-human-FGF-basic-146-aa, R&D systems®, Abingdon, UK), 10 nM dexamethazone (Dexamethazone, Sigma-Aldrich, Brøndby, Denmark), and 0.1 mM L-ascorbic acid-2-phosphate (L-ascorbic-acid-2-phosphate-sesquimagnesium-sal-hydrate, Sigma-Aldrich, Brøndby, Denmark), and injected into the bioreactor. The perfusion rate was decreased to 1 ml/min (i.e., superficial

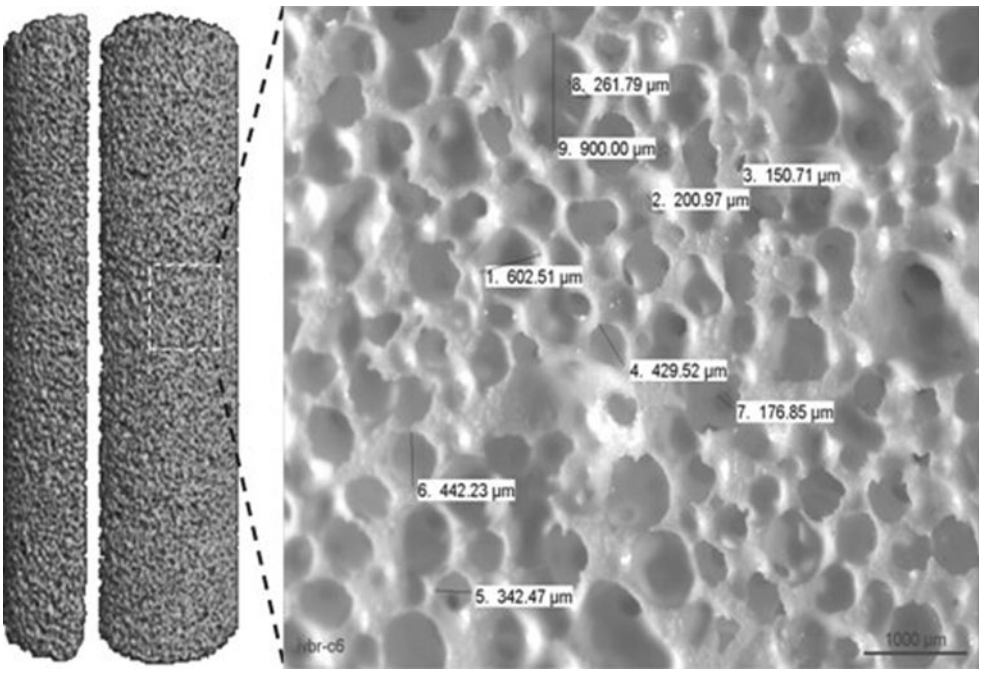

Fig. 2 On the left a 3-D micro-CT image of a non-implanted bone substitute 50 mm long, 15 mm in diameter with a ratio of 7 mm. On the right a stereomicroscopic images of its macroporosity. The diameter of selected macropores is marked with *numbers*

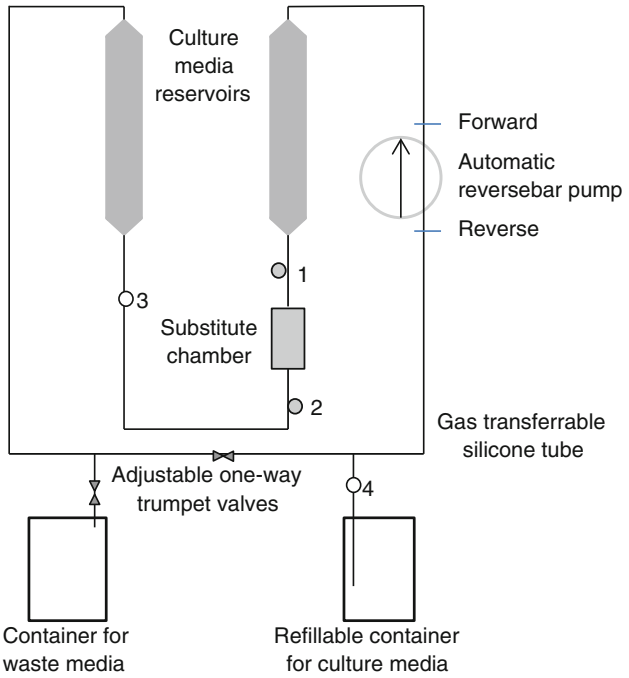

Fig. 3 The bioreactor consists of a substitute chamber between two reservoirs used for the seeding period. The system is driven by an automatic reversebar pump used to pump the media through the substitute chamber in both the seeding and the growth period. The silicone tubing connecting the various parts is permeable to oxygen and CO_2. Oxygen and pH were measured by the use of micro-sensors (PreSens, Precision sensing GmbH, Regensburg, Germany) to observe the viability of the cells (*1–2*). The cells were injected into the perfusion bioreactor through a septum (*3*). Fresh media was applied through another septum (*4*). The system was placed in an incubator at 37 °C in 95 % air/5 % CO_2

velocity of 100 μm/s) and the media was changed twice a week. After 3 weeks of incubation, the substitutes were implanted in the spine fusion animal model.

Surgical procedures

The sheep were premedicated as described previously, anesthetized with 1 mg/kg propofol (Rapinovet, Schering-Plough, Copenhagen, Denmark) giving a total of 55–82 mg pr. sheep and 2 ml of 0.03 mg/ml buprenophine (Temgesic, Schering-Plough, Copenhagen, Denmark) for each sheep. Anesthesia was maintained with 2.5 % isoflurane (Siesta Vet, Dameca, Rødovre, Denmark) and for prevention of infection 4 ml of 250 mg/ml Ampicillin (Ampivet-Vet., Boehringer Ingelheim, Copenhagen Denmark) was injected just prior to the incision and thereafter as prophylactic 2 g ampicillin was injected each day for the next 5 days. For the autograft group, 9–10 g of bone chips were harvested prior to the spine fusion but in the same surgical procedure. Incisions were made over the posterior iliac crest and bone chips were collected with a curved gouge.

The autograft was harvested from the posterior iliac crest and in all but one sheep had additional bone taken from costa 12 to achieve the 9–10 g of bone graft.

The autograft was carefully mixing with the BMNC/α-MEM solution in a petri-dish for a 2 min period in order to achieve a homogenously mass.

A posterior midline incision from costa 12 to the sacrum was made. The muscles were loosened with diathermy and a rougine. The transverse processes, facet joints and corpora were decorticated and all bone segments left in the site. The substitutes or autograft were implanted between the transverse processes close to the corpora. All operations were performed by the same consultant spine surgeon.

As prophylactic pain medicine the sheep were injected three times daily with 2 ml buprenorphine for three initial days. After surgery, the sheep were kept in an observation room for 3 days at the surgical facility with limited access to movement and thereafter taken to a larger confined area. After 4½ months, the sheep were euthanized.

This time-period was based on a pilot study showing that 4½ month follow-up time was appropriate to achieve healing which corresponds to that in patients.

Micro-CT analysis

Each fusion level in the spine segment, L2–L3 and L4–L5, was sawed into a left and right segment due to limitations in the size of the scanning tube of 38 mm. Each segment then contains the fusion mass and transverse processes, but not the central vertebral segment.

The specimens and six non-implanted substitutes were scanned with a high resolution micro-CT scanner (vivaCT40, Scanco Medical AG, Brüttisellen, Switzerland) with a voxelsize of 35 μm using 70 kVp and 84 μA. Micro-CT images were 3-D reconstructed and spine fusion was assessed (Fig. 4).

Definite fusion was defined when bone trabecular were present without interruption between the transverse processes on at least one side of a spine level. An uncertainty was defined as missing a clear single reproducible fusion bridge in a 3D microCT image thus the spine level was considered non-fused.

The microarchitectural characteristics of the fusion mass were estimated using unbiased, true, and assumption-free 3-D methods. In the substitute group the microarchitectural characteristics were calculated from the region of interest including the substitute and the bone inside it. In the autograft group, the microarchitectural characteristics were determined for the fusion mass defined to include the irregular bone above and between the transverse processes excluding the old transverse process bone. The following microarchitectural parameters were quantified: (1) bone density (BV/TV), a measure of the bone content [21];

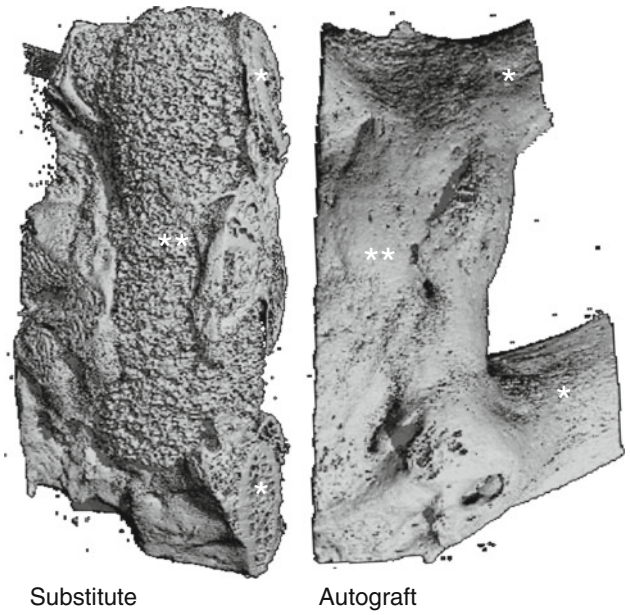

Fig. 4 A complete spine fusion with substitute (*left*) and autograft (*right*) on 3-D micro-CT images of the spine specimens. The images contain the transverse process (*asterisk*) of a fused spine level and the fusion mass (*double asterisks*) between them in one side of a spine level

(2) trabecular thickness (TbTh), calculated from a volume based local thickness of the trabeculae [22]; (3) structure model index (SMI), defines cancellous bone structure based on a differential analysis of the triangulated bone surface. Structure model index = 0 represents an ideal plate-like structure and SMI = 3 represents an ideal rod-like structure. Negative values are caused by very dense cancellous bone structures [23, 24]; (4) the connectivity density (CD) describes the presence of multiple connected trabeculae per volume and calculated using a topological approach [25]; (5) the degree of anisotropy (DA) represents the preferred orientation of the trabecular and the dispersion around this orientation. The parameters from each spine level were added and the mean values used for further analysis.

Statistical analysis

The minimum number of animals was seven based on a frequency of pseudoarthrosis at approximately 20 % using $\alpha = 0.05$ and power of 0.8. The fusion rates were compared with a McNemar's test for correlated proportions. Non-parametric tests were used for comparison of micro-architectural parameters because the data was not normal distributed. The microarchitectural parameters of the implanted and non-implanted substitutes as well as autograft and activated and non-activated substitutes were compared using a Wilcoxon rank sum test. The effect of BMNC/bone marrow aspirate application was analyzed using a Wilcoxon signed rank test. Results were considered statistical significant when $p < 0.05$.

Results

Loss of animals

A total of five sheep were lost during the post-operative follow up period. This was due to surgical complications in the form of deep wound infection, paralytic hind legs, paralytic rumen, and a shoulder injury. The complications were not suspected to be caused by the implanted materials. One sheep in the substitute group was excluded due to a very low number of nucleated cells in the bone marrow aspirate, leaving 12 sheep for final analysis. All included sheep had normal daily activities and the body weight did not differ between groups.

Fusion assessment

From the 3-D micro-CT images, 18 of the 24 spine levels were fused in the autograft- and substitute group. In the substitute group, three of six sheep fused at the levels with both activated and non-activated bone substitute. Two of six sheep only fused at the levels with bioreactor activated bone substitute. One of six sheep did not fuse at any level. Thus, five levels fused when the bone substitutes were activated in the bioreactor, whereas only three fused without activation ($p = 0.25$).

In the autograft group, three of six sheep fused at the levels with autograft alone and autograft supplemented with BMNC. One of six sheep did not experience fusion at any level.

Due to the loss of animals there were an unequal number of spine levels with autograft alone and autograft supplemented with BMNC. Four sheep had autograft supplemented with BMNC at L5–L6 whereas only two sheep with BMNC at spine level L2–L3.

Micro-CT evaluation

The microarchitectural parameters of the bioreactor activated substitute were compared to the non-activated substitute and a higher bone density ($p = 0.028$) and degree of anisotropy were found in the bioreactor activated substitute ($p = 0.028$). No difference existed on the other parameters (Fig. 5).

When both the bioreactor activated and non-activated bone substitute were compared to non-implanted bone substitute (Fig. 5), the bone density and trabecular thickness of both implanted substitutes were increased compared to non-implanted substitute ($p = 0.005$); The SMI of

both implanted substitutes was significantly lower than that of non-implanted substitutes indicating a change from a combined plate and rod like structure to a more plate like structure ($p = 0.005$); No difference in CD between both implanted substitutes and non implanted substitutes were found ($p > 0.50$); The DA of implanted substitutes were reduced compared to non-implanted substitutes ($p = 0.005$).

When the microachitecture of the bone structure in autograft alone and autograft supplemented with BMNC

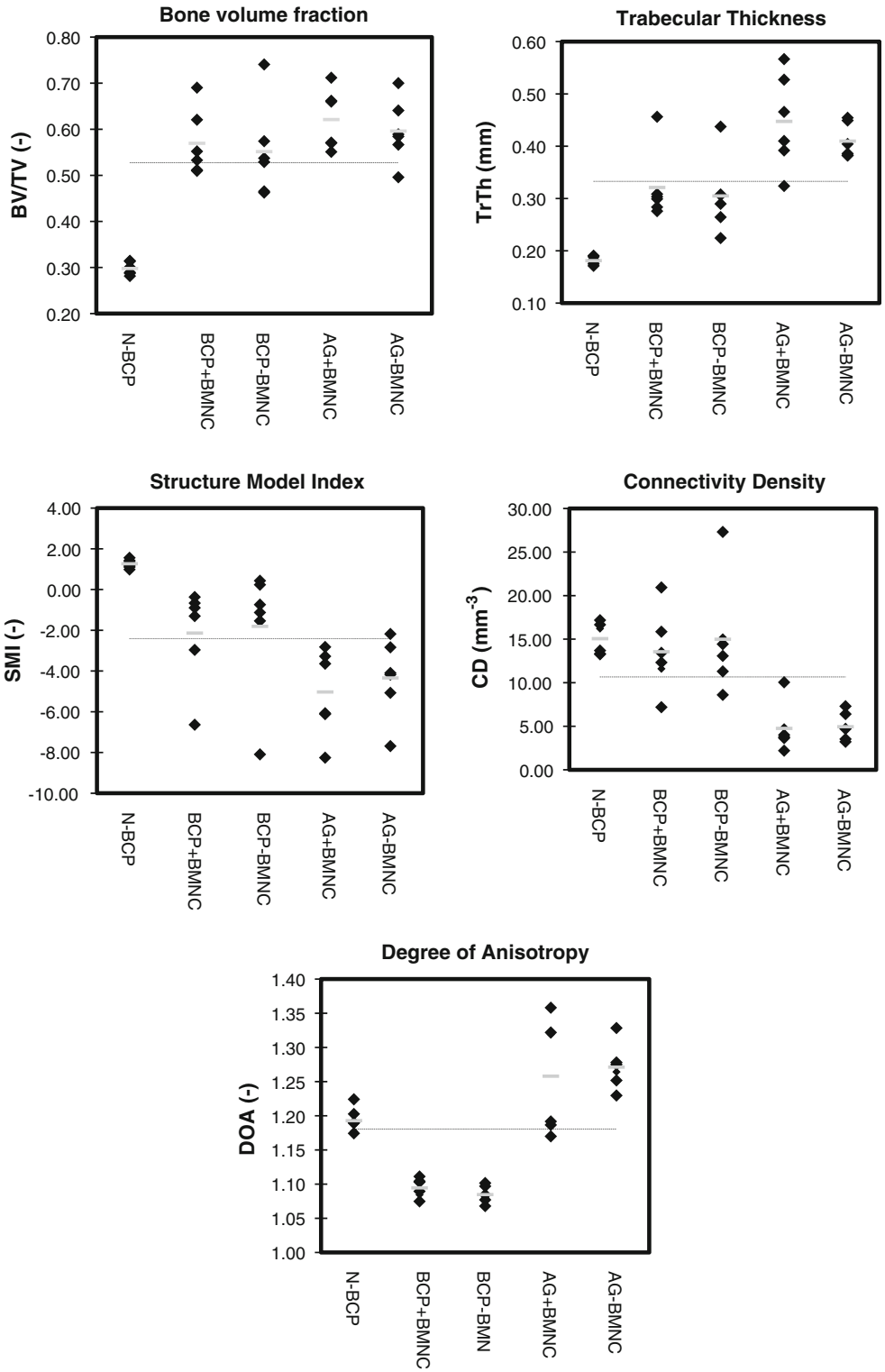

Fig. 5 Data for bone density (BV/TV), trabecular thickness (TbTh), structure model index (SMI), connectivity density (CD) and degree of anisotropy (DA) are shown. The figures are of non-implanted substitute (N-BCP), activated or non-activated substitute (BCP ± BMNC) and autograft with and without bone marrow aspirates (AG ± BMNC)

were compared no difference was found on any of the parameters (Fig. 5). Therefore the autograft group was combined to one average value for each sheep and compared to bioreactor activated and non-activated bone substitutes (Fig. 5).

The bone density was significantly increased in the autograft group compared to both bioreactor activated ($p = 0.03$) and non-activated bone substitutes ($p = 0.008$). The trabecular thickness was likewise significantly increased in the autograft group compared to the bioreactor activated ($p = 0.03$) and non-activated substitutes ($p = 0.02$). No difference was found between the SMI of the autograft and bioreactor activated ($p = 0.07$) and non-activated bone substitutes ($p = 0.07$). The CD was significantly lower in the autograft group compared to bioreactor activated ($p = 0.005$) and non-activated substitute ($p = 0.005$). The DA was significantly increased in the autograft group compared to bioreactor activated ($p = 0.005$) and non-activated substitutes ($p = 0.005$).

Discussion

The study is unique by including large bioreactor activated cell-based bone substitutes assessed in a large clinical relevant animal model. Previous investigations have used substitutes in perfusion bioreactors but they have not been tested in large animal models [26, 27].

Fusion efficacy

The fusion rate was 83 % (five of six spine levels) in bioreactor activated substitutes and 50 % (three of six spine levels) in substitutes without activation. In the autograft group a fusion rate of 83 % (five of six spine levels) was observed with no effect of BMNC.

The study indicates that the fusion efficacy of bioreactor activated substitutes were better than that of non-activated bone substitutes and that it might be similar to the fusion efficacy of autograft. This might be due to the combined osteogenic properties of the BMNC and the osteoconductive properties of the highly porous BCP.

Unfortunately five sheep were euthanized early and one was excluded due to a low total cell number leaving only six sheep in each group instead of the intended nine sheep in each group why the results have to be interpreted with caution.

Bone formation in bone substitute

The bone density of the overall implanted substitutes increased to almost twice the value of the density of non-implanted substitutes. This demonstrated a substantial bone formation inside the bone substitute, thus indicating that the PLA reinforced BCP had osteoconductive properties. Similar to bone density, the trabecular thickness of the bone-substitute was increased after implantation of the substitute, which indicates mineral deposition on the existing walls of the bone substitutes. The bone volume of the cell-activated bone substitute also increased but this difference to small to be of clinical relevance.

No difference in the SMI values was found between the activated and non-activated bone substitutes. The structure of the implanted substitutes changed when compared to non-implanted substitute from a combination of plates and rods towards a typical plate-like structure with fenestrations of the concave walls as indicated by the negative SMI. The plate like structure strengthened the spine fusion, as a previous study demonstrated that plate-like structure reflects high mechanical strength, compared to rod-like structure that reflects low mechanical strength [28].

The CD was not significantly changed after implantation of the substitutes and no difference was found between activated and non-activated bone substitutes.

After implantation, the activated substitutes were more isotropic than non-activated substitutes. The degree of anisotropy between implanted and non-implanted bone substitutes was significantly reduced to a more isotropic structure with a degree of anisotropy of 1.10 in the implanted substitute group. It could be explained by new bone mineral deposition in a different pattern as the bone substitute was being resorbed.

Bone structure of bone substitute compared to autograft

There is a significant higher bone density and trabecular thickness in the autograft group compared to both activated and non-activated bone substitutes. This indicates that though the activated substitute might have a similar fusion efficacy to autograft, the fusion bridge is not of equal substance. This might be due to the initial high porosity of the substitute, which cause a lower bone density compared to autograft. The autograft group had a lower CD and higher DA than the activated and non-activated substitutes. This indicates that the autograft is less isotropic and more compliant with the actual bone stress levels than the substitute groups.

Conclusion

We found that bioreactor-generated cell-based bone substitutes seemed superior in fusion ability when compared to cell-free bone substitute and comparable to autograft in fusion ability, but not in bone structure. This combined with the favorable biocompatible abilities and strength comparable to human cancellous bone indicates that it

might be a suitable bone substitute in spine fusion procedures.

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Conflict of interest None.

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