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RESEARCH ARTICLE

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Autologous blood coagulum is a physiological carrier for BMP6 to induce new bone formation and promote posterolateral lumbar spine fusion in rabbits

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Abstract

In the present study, we describe autologous blood coagulum (ABC) as a physiological carrier for BMP6 to induce new bone formation. Recombinant human BMP6 (rhBMP6), dispersed within ABC and formed as an autologous bone graft substitute (ABGS), was evaluated either with or without allograft bone particles (ALLO) in rat subcutaneous implants and in a posterolateral lumbar fusion (PLF) model in rabbits. ABGS induced endochondral bone differentiation in rat subcutaneous implants. Coating ALLO by ABC significantly decreased the formation of multinucleated foreign body giant cells (FBGCs) in implants, as compared with ALLO alone. However, addition of rhBMP6 to ABC/ALLO induced a robust endochondral bone formation with little or no FBGCs in the implant. In rabbit PLF model, ABGS induced new bone formation uniformly within the implant resulting in a complete fusion when placed between two lumbar transverse processes in the posterolateral gutter with an optimum dose of 100-µg rhBMP6 per ml of ABC. ABGS containing ALLO also resulted in a fusion where the ALLO was replaced by the newly formed bone via creeping substitution. Our findings demonstrate for the first time that rhBMP6, with ABC as a carrier, induced a robust bone formation with a complete spinal fusion in a rabbit PLF model. RhBMP6 was effective at low doses with ABC serving as a physiological substratum providing a permissive environment by protecting against foreign body reaction elicited by ALLO.

KEYWORDS

allograft (ALLO), autologous blood coagulum (ABC), autologous bone graft substitute (ABGS), foreign body giant cells, posterolateral lumbar fusion (PLF), recombinant human BMP6 (rhBMP6)

1 | INTRODUCTION

Spinal fusion surgery is commonly performed in patients, where two or more adjacent vertebral segments are fused to restrict motion primarily to relieve the source of back and leg pain (Bohl et al., 2015; Diebo et al., 2015; Gupta, Mohan, & Gupta, 2017; Mobbs, Loganathan, Yeung, & Rao, 2013; Mobbs, Phan, Malham, Seex, & Rao, 2015). Autografts and allografts containing autologous bone marrow are routinely employed to stimulate osteogenesis either at the intervertebral space as in anterior lumbar interbody fusion (ALIF; Bohl et al., 2015) or at an ectopic site between two lumbar transverse processes bilaterally as in a posterolateral lumbar fusion (PLF; Liu, Wang, Qiu, Weng, & Yu, 2014). A variety of disorders may be treated with spinal fusion, including degenerative disc disease (DDD), spondylolisthesis, spinal stenosis, scoliosis, infections, spinal fractures and dystrophy, and various tumours (McAnany et al., 2016).

Autograft from the patient's posterior iliac crest bone is the "gold standard" for spine fusion surgery as the harvested bone chips have live bone marrow cells and an immunologically compatible extracellular matrix (Garcia-Gareta, Coathup, & Blunn, 2015; Goldberg & Stevenson, 1987; Tilkeridis et al., 2014). However, the use of autograft presents several disadvantages: (a) It requires another incision that may result in postoperative pain and an increased risk for infection and (b) the amount of bone that can be harvested is limited (Fernyhough, Schimandle, Weigel, Edwards, & Levine, 1992; Murphy et al., 2019). As an alternative to autograft, several compositions are employed with modest outcome that include allograft (cadaver bone from a bone bank), demineralized bone matrix (Cahill, Chi, Day, & Claus, 2009; Hsu, 2014), various ceramics (calcium-based compounds) in conjunction with patients bone marrow (Carragee, Hurwitz, & Weiner, 2011), and bone morphogenetic proteins (BMPs) in combination with animal derived collagen (Simmonds et al., 2013) and/or with ceramic composite scaffolds (Brown et al., 2013).

Recombinant human BMP2 (rhBMP2) applied within an absorbable collagen sponge (InFUSE) has been approved to treat DDD at one level fusion (vertebra-disc-vertebra; ALIF) from L2 to S1 using Titanium LT cages via an anterior (ALIF) and with polyetheretherketone (PEEK) cages via lateral (oblique lateral interbody fusion) approach in skeletally mature patients (Carragee et al., 2011). An off-label use of INFUSE in related interbody fusion procedures (cervical) has resulted in unwanted safety issues, presumably from the high rhBMP2 dose employed (Brown et al., 2013; Hsu, 2014; Jain, Hassanzadeh, Strike, Skolasky, & Riley, 2014; Riederman et al., 2017; Simmonds et al., 2013). However, the clinical evaluation of rhBMP2 soaked in synthetic ceramics (hydroxyapatite and tricalcium phosphate) and bovine-sourced collagen composite as a scaffold (Amplify) for the posterolateral lumbar spinal fusion procedure (Glassman et al., 2008) has not been approved by Food and Drug Administration for human use, in part due to potential cancer risks in treated groups as compared with autograft controls (Carragee et al., 2013) and posed numerous challenges including unwanted safety issues likely resulting from the high dose of rhBMP2 employed (12–40 mg for a single-level fusion; Brown

et al., 2013; Cahill et al., 2009; Carragee et al., 2011; Fu et al., 2013; Hsu, 2014; Simmonds et al., 2013; Vukicevic et al., 2014; Vukicevic & Sampath, 2017). Similarly, rhBMP7 that contained bovine bone collagen dispersed with additive carboxyl-methyl cellulose (OP-1 Putty) has also failed to achieve successful outcome (Vaccaro et al., 2008). These data suggest that use of low doses of a BMP with a natural biocompatible scaffold may provide a more permissive environment for the optimal bone formation that is restricted to the implant site. Here, we demonstrate that an autologous bone graft substitute (ABGS) that contains a low dose of rhBMP6 that has a low affinity for the endogenous BMP antagonist, Noggin (abundant in bone; Song et al., 2010), delivered with an autologous blood coagulum (ABC) carrier with or without bone allograft particles (ALLO), is capable of inducing new bone formation in rat subcutaneous implants and achieving a complete fusion in rabbit PLF model.

2 | MATERIALS AND METHODS

2.1 | Rat subcutaneous implant assay analyses

Rat subcutaneous assay was carried out to observe cellular events and bone formation following subcutaneous implantation of allograft, ABC, and rhBMP6 (Sampath & Reddi, 1981). Three types of implants (ALLO, ABC + ALLO, and ABC + ALLO + rhBMP6) were composed and implanted in the axillary region of male Sprague-Dawley rats, aged 10 weeks. ABGS was prepared from 0.5 ml of rat blood, which was mixed with 0.1 mg of allograft and 25 μg of rhBMP6 and left for 60 min to coagulate in a 1-ml syringe. After removing the serum, the ABGS was implanted. Two different experiments were performed and implants were harvested on Days 1, 3, 7, and 14 in the first and on Days 7 and 35 in the second experiment, with two rats and four implants for each time point. Bone formation and cellular events were analysed using micro-CT and histology sections.

2.2 | Rabbit model

Study protocols were conducted in 14-week-old male New Zealand White laboratory rabbits (*Oryctolagus cuniculus*), New Zealand strain, body weight 3–5 kg. The animal facility was registered by Directorate for Veterinary, Reg. No: HR-POK-001. An approval for the animal studies was given by the Directorate for Veterinary and Food Safety, Ministry of Agriculture, Republic of Croatia (approval No. 525-10/0255-14-3). Laboratory animals were housed in standard rabbit cages in conventional laboratory conditions at the temperature of 18–22°C, relative humidity of 50–70%, fluorescent lighting provided illumination 12 hr/day and noise level 60 dB. Standard diet (Mucedola, Italy) bedding with environmental enrichment were available, and fresh water was provided ad libitum. Animal care was in compliance with standard operating procedures of registries Croatian Animal facility HR-POK-001; using 3R principle, minimization of the pain suffering during the experiment; the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

2.3 | ABGS implant preparation

ABGS without ALLO was produced by dispersing rhBMP6 into autologous blood, which then allowed to form coagulum with defined structure and rheological properties as determined by stiffness, elasticity, and strain, testing as previously described (Grgurevic et al., 2018). Blood samples were collected from rabbit marginal ear veins into tubes without any anticoagulant substance, supplemented with 0.1 ml of 50-mM $CaCl_2$ solution in a volume of 2.5 ml, 2 hr before surgery, and lyophilized rhBMP6 (Genera Research, Croatia) was dissolved in water for injection (c = 2.5 mg/ml) and mixed with blood in an appropriate volume and then left in room temperature to coagulate. Allogenic devitalized bone particles (ALLO) were prepared as described (Tomford, 2000), and 70- to 420-μm particle sizes were used to produce ABGS with allograft. To produce ABGS with ALLO, ALLO particles were added at 0.2 g/ml ABC/rhBMP6 mix and then left at room temperature to coagulate. In biomechanical testing, amounts of 1, 1.5, and 2 g of ALLO per 5 ml of ABC/rhBMP6 were used.

2.4 | PLF operation procedure

Twenty-eight skeletally mature rabbits underwent bilateral posterior intertransverse process fusion between lumbar vertebrae L4 and L5 (Boden, Schimandle, & Hutton, 1995). Animals were assigned into seven experimental groups with N = 4 in each group as follows: ABC alone; ABC and ALLO; ABC with 50 μg/ml rhBMP6; ABC with 100 μg/ml rhBMP6; ABC with 200 μg/ml rhBMP6; ABC with 200 μg/ml rhBMP6 and ALLO; and ABC with 400 μg/ml rhBMP6 and ALLO.

The operations were carried out under general anaesthesia. Xylazine (Xylapan®, Vetoquinol, Switzerland) in dose of 5 mg/kg body weight and Ketamine (Ketaminol® Vetoquinol, Switzerland) in dose of 35 mg/kg body weight were applied intramuscularly. Analgetic ketoprofen (Ketofen®, Merial, France) in dose of 4 mg/kg body weight was applied s.c. Prior to iv catheter placement hair was clipped and skin aseptically prepared. Spinal fusion was carried out in the lumbar region between L4 and L5 vertebrae. After placing the rabbit in the prone position, a dorsal midline skin incision extending from L4 to L7 was made followed by a paramedian fascial incision (Boden et al., 1995; Schimandle, Boden, & Hutton, 1995). An intermuscular plane was established between the multifidus and longissimus muscle layers using blunt dissection facilitating exposure of the transverse processes of L5 and L6 as well as the intertransverse membrane. An electric cauterizer was used as needed to minimize blood loss. Defects (device was placed between the transverse process in the paraspinal bed bilaterally) were filled with ABC alone or in combination with ALLO and rhBMP6 according to the predefined experimental groups. Lateral aspect of transverse processes were decorticated until bleeding by high speed burr where prepared ABGS devices were placed. The fascial incision was closed with 4-0 synthetic glycolide/lactide copolymer absorbable sutures. The aforementioned procedure was repeated on the contralateral side.

Whereas in the first experiment, decortication of transverse processes was performed as an usual procedure in all the operations, in a separate experiment bone, decortication was explored as a contributing factor to the quality of the spinal fusion (Ishikawa, Shin, Bowen, & Cummings, 1994). Twelve animals were divided into three groups with N = 4 in each group as follows: ABC with 100 μg/ml rhBMP6 with decortication, ABC with 100 μg/ml rhBMP6 without decortication, and ABC with 100 μg/ml rhBMP6 and ALLO without decortication.

All animals were euthanized 14 weeks after the surgery by using premedication of 3 mg/kg xylapane and 20 mg/kg ketamine i.m. and administration of T61 (1 ml/kg) i.v. Ethical principles of the study ensured compliance with European Directive 2010/63/EU, the Law on Amendments to Animal Protection Act (Official Gazette 37/13), the Animal Protection Act (Official Gazette 102/17), Ordinance on the protection of animals used for scientific purposes (Official Gazette 55/13), FELASA recommendations, and recommendations of the Ethics Committee School of Medicine, University of Zagreb. During experiment, no adverse effects have been observed in any of the experimental groups.

2.5 | Anti-rhBMP6 antibodies

The presence of anti-rhBMP6 antibodies was investigated within the toxicology GLP study on BMP6 biocompatibility, safety, and efficacy, including biochemical, hematological, gross pathology, and histology examination carried out in Meditox, Czech Republic, as one aspect of this study. The toxicology study has been conducted in 30 rabbits and was approved by Meditox s.r.o. Institutional Animal Care and Use Committee and the Committee for Animal Protection of the Ministry of Health of the Czech Republic (58/2016). New Zealand white rabbits were used, n = 5/sex/dose level (total of 30 animals). Three dose groups were administered with 0 (control, ABC alone), 1 mg (low dose), and 2-mg rhBMP6/ABC (high dose) per animal with surgical spinal implantation between vertebrae L4 and L5 as described above. Transverse processes of L4 and L5 were decorticated with high speed burr, and area between them was filled with ABC alone (control) and ABC with 2 × 0.5 mg of rhBMP6 for low-dose group and ABC with 2 × 1 of mg rhBMP6 for high-dose group. Application volume for all groups was 2 × 2.5 ml per animal. Blood samples for antibody assay were collected during the Week 1 and on Day 21 from the *v. auricularis* or *v. saphena* into tubes without anticoagulant. Samples were centrifuged (6,000 rpm for 10 min), and the serum split into aliquots and immediately frozen at −80°C until analysis.

2.6 | Methods of evaluation

Radiographical images were taken before the surgery and at Weeks 3, 6, 9, and 14 after surgery. X-ray imaging of lumbar spine segment were performed using two standard orthogonal views (lateral and dorsoventral). Samples were scanned on Eichermeyer EDR HP (IMD Generators s. r. l., Italy) X-ray machine using the 40 kV and 8 mAs with all ionization protection protocols respected during the imaging, whereas images were processed using Agfa CR 30-X (Agfa, Japan). All obtained radiographs from rabbit bones were interpreted and scored using a

radiographic grading score system (Lindley et al., 2017) by a surgeon and a radiologist blinded to the treatment protocol and postoperative interval.

Micro-CT detailed analysis of rabbit lumbar spine spanning from L4 to L7 was done using the Sky Scan 1076 micro-CT device (Hildebrand, Laib, Muller, Dequeker, & Ruegsegger, 1999). Ex vivo lumbar spine was scanned at the resolution of 18 μm, 0.5-mm aluminium filter, 0.5° rotational step, and frame averaging set at 2. Concurrent dataset analysis of the site of implantation was analysed by CTAn (Sky Scan) software. The new bone formation and trabecular bone parameters were depicted throughout the whole area of newly formed bone, as previously described (Erjavec et al., 2016; Grgurevic et al., 2011).

2.7 | Histology

Explanted rat subcutaneous samples were fixed in 4% formalin for 10 days. After fixation, samples from Days 7 and 14 were decalceinated using 14% EDTA in 4% formalin solution for 20 days, with solution change every 2 days. All samples were embedded in paraffin and cut at 5-μm slice thickness. To identify progenitor cells present preceding to and during bone formation, sections were stained with alkaline phosphatase, and the cell numbers were expressed per mm^2 of the implant area. For measurement of the inflammatory response in the rat subcutaneous implant model, slices were subjected to analysis of foreign body giant cells (FBGCs) by H&E staining and acid phosphatase detection by histochemistry. Number of FBGC was determined on histological sections (three to four samples per group), and on each sample, FBGCs were counted in three fields (one field = 1 mm^2). Immunohistochemistry of rat subcutaneous implants was performed using the Mouse and Rabbit specific IHC Detection kit—Micropolymer (Abcam ab236467) with following antibodies: anti-alkaline phosphatase, dilution 1:250 (Abcam ab108337); anti-Sox9, dilution 1:2,000 (Abcam ab185230); and anti-osteocalcin, 10 μg/ml (Abcam ab13418).

Muscle free rabbit spine samples were fixed in 4% formalin for 3 weeks, trimmed of soft tissue, and entire bone was embedded in plastic resin (Technovit 7200). Samples were cut at 5-μm slices with a diamond saw and stained using Masson Goldner Trichrome dye, as previously described (Krempien, Vukicevic, Vogel, Stavljenic, & Buchele, 1988; Vukicevic, Krempien, & Stavljenic, 1987). Images were obtained using Olympus BX51 Epi-Fluorescence microscope.

2.8 | rhBMP6 immunochemical analyses

Time-dependent release of rhBMP6 from the ABC plus allograft in vitro was determined on blood samples from healthy human volunteers. Blood was collected from the cubital vein into tubes without anticoagulants. Upon withdrawal, blood was mixed with allograft (particle sizes 2–5 or 5–8 mm) and rhBMP6 in two concentrations (62.5 or 125 μg). After the coagulation was completed (60 min), ABC+rhBMP6 plus allograft was rinsed with 1 ml of the basal medium. Each implant was placed in a Falcon tube containing 3 ml of Dulbecco's modified Eagle medium. Tubes were incubated at 37°C during 10 days, and the medium was replaced on Days 1, 3, 6, 8, and 10. The amount of BMP6 released from the ABC/ALLO/rhBMP6 in the medium was determined by rhBMP6-specific ELISA (R&D systems, DY507).

For determination of the presence of anti-rhBMP6 antibodies in the serum samples, a previously validated indirect ELISA method was used. Microtiter plates were coated overnight with 100 ng/ml rhBMP6 diluted in carbonate buffer. Serum samples were diluted in reagent diluent (1% BSA in PBS) to six different dilutions (1:50, 1:100, 1:500, 1:1,000, 1:5,000, and 1:10,000), and biotinylated goat anti-rabbit IgG (R&D BAF008, diluted 1:5,000) was used as secondary antibody. Serum samples from six animals were analysed on the same plate, and each plate included the negative serum pool diluted at the same ratio as the study samples. Absorbance was read at 450 nm using Absorbance Microplate Reader ELx 808TM. Validation cut point value for each dilution point was determined during the method validation (Mire-Sluis et al., 2004). Briefly, 10 samples of rabbit naïve serum were analysed in dilution series from 1:50 to 1:10,000. The assay cut point was calculated according to the following formula: mean absorbance + 1.645 × SD, where 1.645 is the 95th percentile of the normal distribution. Samples with absorbance readings higher than determined cut point value at particular dilution would be defined as positive.

2.9 | Data management

Values are expressed as mean ± SEM or SD as indicated. For statistical comparison of two samples, a two-tailed Student t test was used, and $P < .05$ was considered significant where indicated. Two-way analysis of variance with Duncan's multiple range test was performed to determine the effect of treatment and time on biochemical and bone repair parameters. Additional specific data analyses, if applicable, are presented in figure legends. Analyses were performed by SAS for Windows 9.3 (SAS Inc.).

3 | RESULTS

3.1 | Bio-responsiveness by ABGS in rat subcutaneous assay

Histology of ALLO/ABC and ALLO/ABC/rhBMP6 implants on Days 7 and 35 is shown in Figure 1a. There was no bone formation in ALLO/ABC implants (top panel). On the other hand, ABC/ALLO/rhBMP6 implants showed endochondral bone formation (bottom panel). In ABC/ALLO/rhBMP6 Day 7 implants, we further confirmed the newly formed cartilage with histochemical staining for SOX-9, a transcription factor for chondrogenesis (Figure S1A,B), and the evidence of new bone formation by alkaline phosphatase (Figure S1C) and osteocalcin (Figure S1D) staining, markers of osteoblast phenotype. The newly formed bone underwent a typical bone remodelling mediated by osteoclasts as shown on Day 35 implants, where ALLO particles were replaced by newly formed bone via a creeping substitution (Figure 1a, bottom panel). The robust osteogenesis was observed with a gradual resorption of ALLO bone particles, as visualized by
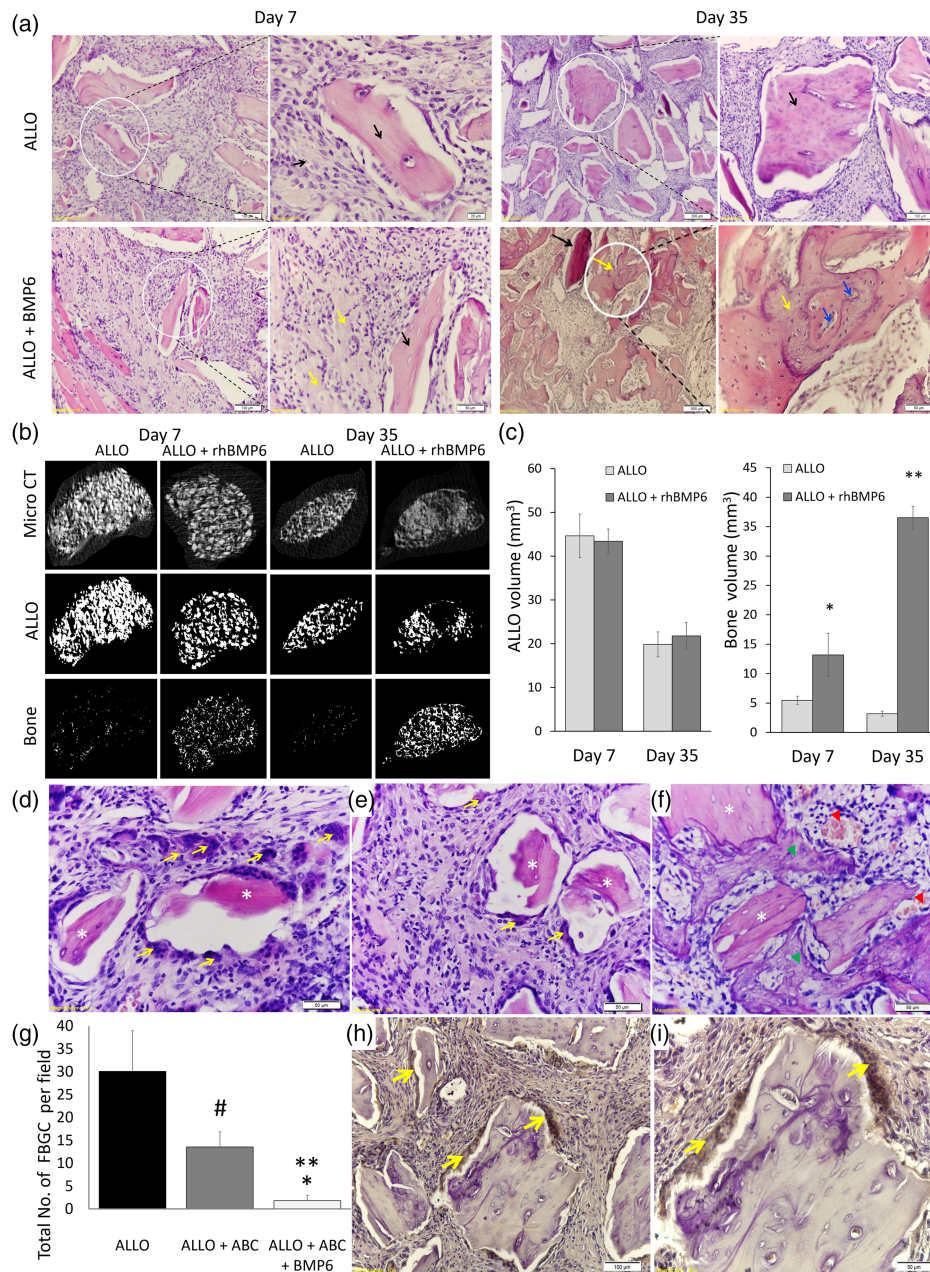
FIGURE 1 Allograft in ABC without and with rhBMP6 following subcutaneous implantation in rats. (a) ABC/ALLO (black arrows) without rhBMP6 induced formation of fibrotic tissue at Days 7 and 35 without any sign of new bone formation. ABC/ALLO with rhBMP6 induced new bone formation at Day 7 (yellow arrows) and advanced creeping substitution of ALLO with new bone was observed on Day 35 (blue arrowhead). (b) Overall micro-CT analyses of ABC/ALLO implants without and with rhBMP6 are shown in the top row, only ALLO particles are visualized in the middle row, and the bottom row represents the newly formed bone, image obtained upon subtracting the ALLO particle from overall micro CT. Note the formation of new bone by 7 days and significant by Day 35 in ABC/ALLO implants that contained rhBMP6. ABC/ALLO alone did not induce bone either at 7 or 35 days after implantation. (c) Morphometric analysis of ALLO volume in implants on Days 7 and 35 indicating a significant decrease of ALLO volume and increased of the amount of bone in the presence of rhBMP6. Results are shown as mean ± SD (n = 5). *P < .05 and **P < .01 versus ALLO (two-tailed Student's t test). (d) ALLO (white asterisks) when implanted without ABC resulted in recruitment of numerous foreign body giant cells (FBGC; yellow arrows). (e) ALLO when implanted with ABC showed significantly decreased number of FBGCs. (f) ALLO when mixed with ABC and rhBMP6 induced endochondral and intramembranous bone formation (green arrowheads) in between ALLO particles and surrounded by new vasculature (red arrowheads), note absence of FBGCs. (g) Total number of FBGCs from three histological sections of representative implants. *P < .05 and **P < .01 versus ALLO and ALLO + ABC, respectively; #P < .05 versus ALLO and ALLO/ABC/rhBMP6 (two-tailed Student's t test). (h,i) Multinucleated FBGCs stained for acid phosphatase (yellow arrows) in adjacent sections of ALLO alone implants

contrast micro-CT analysis (Figure 1b). ALLO volume and bone volume in the rat subcutaneous implants were quantified using micro-CT analysis for ABC/ALLO and ABC/ALLO/rhBMP6 implants on Days 7 and 35 (Figure 1c).

When implanted in rat subcutaneous sites, ALLO bone particles induced inflammation and foreign-body reaction due to high Ca/P mineral content at ectopic sites by recruiting mononuclear phagocytes by Days 1–3 (data not shown), which then fused to form multinucleated FBGCs by Days 7–14 (Figure 1d). The fusion of multinucleated FBGCs was significantly reduced when ALLO particles were formulated with ABC (Figure 1e). In the ABGS implants that contained ABC/ALLO/rhBMP6, there were even fewer or no FBGCs, and new endochondral bone formation was observed in apposition to ALLO particles (Figure 1f). Figure 1g represents the average number of multinucleated FBGCs counted morphometrically from three representative histology sections from ALLO, ALLO/ABC, and ALLO/ABC/rhBMP6 implants. The multinucleated FBGCs cells recruited by ALLO implants were further characterized by immuno-histochemistry for acid phosphatase staining, which was reduced by addition of ABC (Figure 1h,i).

3.2 | Production of ABGS device

The shape and dimension of the produced ABGS implant for use in rabbit PLF model is presented in Figure 2a. In ABGS/ALLO implants, the added ALLO particles were distributed uniformly within the implants, as revealed by X-ray and micro-CT analysis. The presence of ALLO has significantly increased the stiffness and elasticity of the device and improved compatibility and handling properties as compared with the implants without ALLO (Figure 2b). About 0.1 to 0.2 g of ALLO particles (74–420 μM) per 0.5 ml of ABC is sufficient to provide the handling rheological properties.

As rhBMP6 is mainly bound to plasma proteins in ABGS (Grgurevic et al., 2018), the calculated cumulative total release measured in vitro over 10 days from ABGS was only 3–5% of total rhBMP6 dose (Figure 2c). Addition of ALLO in different particle sizes seemed to slightly attribute to more cumulative release. The release kinetics of rhBMP6 may change at the implant site as the protein is taken up by the responding cells to trigger endochondral bone differentiation as well as fibrinolysis of ABGS, which does not occur in vitro. Furthermore, it is noteworthy that there were no anti-rhBMP6 antibodies detected in sera of rabbits treated with ABC/rhBMP6 implants (1 and 2 mg of rhBMP6 per animal) at 3 weeks following implantation (Figure 2d).

3.3 | Evaluation of ABGS in rabbit PLF model

The follow-up of the new bone formation in all experimental groups as assessed by X-ray at different time points after surgery is shown in Figure 3. Figure 4a shows X-ray, micro-CT, and gross anatomy photographs of ABGS without and with ALLO at varying doses of rhBMP6 per ml of ABC as compared with ABC alone and ABC plus ALLO groups at the end of experiment, 14 weeks after surgery. Bone

decortication of transverse processes was performed in all the groups. The group that contained rhBMP6 at 50 μg/ml ABC demonstrated new bone formation but achieved fusion in only two out of four rabbits, suggesting that the amount of rhBMP6 might not have been sufficient, whereas rhBMP6 at 100 μg/ml ABC achieved a complete spinal fusion in all the rabbits. Because ALLO is capable of inducing inflammation and formation of multinucleated FBGCs, we have formulated ABGS implants with higher amounts of rhBMP6. ABGS/ALLO that contained 200 μg/ml ABC induced a complete fusion with bone volume comparable with that of ABGS that had 100 μg/ml ABC without ALLO; however, an increased amount of rhBMP6 to 400 μg/ml ABC did not further increase the bone volume. In the groups that had ABC alone or ABC/ALLO, no bone formation was observed and spine did not fuse. The successfully fused bone by ABGS (ABC/rhBMP6 or ABC/ALLO/rhBMP6) groups appeared to be compact and solid (Figure 4a). The contact area between the transverse processes and the newly formed bone was indistinguishable and fused into one continuous bone segment.

Radiographic images of all samples were scored and measurement of new bone was quantified for bone volume, trabecular number and trabecular interconnectivity, as determined by micro-CT analyses and are presented in Figure 4b–e. ABGS at a dose of 100-μg rhBMP6 per ml of ABC appears to be the optimal dose, and doubling the dose does not increase the bone formation parameters. ABGS plus ALLO implants that contained either 200- or 400-μg rhBMP6 per ml of ABC resulted in new bone formation comparable with ABGS without ALLO at 100-μg rhBMP6 per ml of ABC.

The histology showed that in ABGS implants, the newly formed bone underwent a new bone formation with a typical remodelling and osseous integration at the interface in between the newly formed bone and the native transverse processes (Figure 5). In ABGS containing ALLO implants, the newly formed bone underwent a rapid bone remodelling and was fully integrated with ALLO particles, which eventually got replaced by creeping substitution. An extended osteoid seam covered surfaces of newly formed bone with areas of woven bone and immature osteocytes (Figure 5).

In addition, we examined whether decortication of transverse processes is required to achieve a successful fusion. Rabbits were treated with implants ABC containing 100-μg rhBMP6 per ml with or without decortication during the operation procedure. The results show that two out of four implants did not fuse rabbits without decortication procedure, whereas four out of four implants have fused in rabbits upon decortication. We also found ABGS when formulated with ALLO, three out of three implants have fused in rabbits without decortication, suggesting that ALLO may facilitate the fusion of transverse processes with newly formed bone with or without decortication (Figure 6).

4 | DISCUSSION

In the present study, we demonstrated that an ABGS that contains rhBMP6 dispersed within ABC, without or with ALLO particles, is
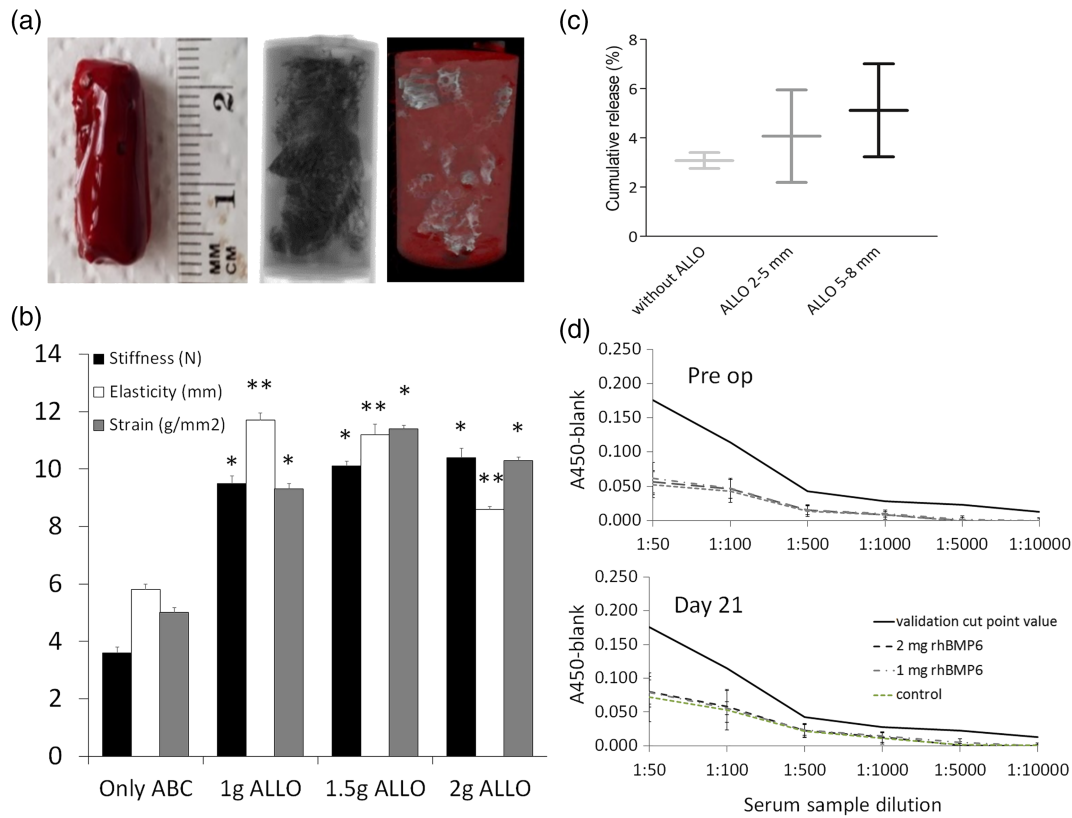
(a)

(b)

(c)

(d)

FIGURE 2 The shape and dimension of the produced ABGS implants for use in rabbit PLF model. (a) ABGS implants with allograft prepared for rabbit (1.5 × 1.0 cm) posterolateral spine fusion experiments; left is gross picture, the centre is X-ray, and right is micro-CT. (b) Biomechanical properties of ABGS were tested using the CUT test for measuring stiffness, elasticity, and strain in implants with various allograft amounts (1–2 g) per 5 ml of blood. Values are shown as mean ± *SEM* (*n* = 4), $^*P$ < .05 and $^{**}P$ < .01 (two-tailed Student's *t* test) when compared with the implant without allograft. (c) Cumulative in vitro release of rhBMP6 over 10 days from ABGS expressed as a percent of the total rhBMP6 dose. Results are presented as individual values from two experiments. (d) Absence of the anti-rhBMP6 antibodies in rabbit serum was demonstrated by indirect ELISA method a week before and 3 weeks after surgery. Cut point value was determined during the method validation process. Mean ± *SD* (*n* = 10) of absorbance (A_{450}) values are shown

capable of inducing new bone formation in rat subcutaneous implants and achieving a fusion in a rabbit spine PLF model. BMP6 was chosen as a preferred BMP as its binding to Noggin, a natural BMP antagonist present in abundance in bone, is lesser than other BMPs (Klineberg et al., 2014; Song et al., 2010; Vukicevic et al., 2014). BMP6 also binds to most of the Types I and II BMP receptors and exhibits a high specific alkaline phosphatase activity in osteoblastic cell cultures (Grgurevic et al., 2018; Song et al., 2010), hence permitting the use of lower doses as compared with BMP2 or BMP7. ABC was chosen as a physiological carrier as it suppresses foreign body response, promotes tight rhBMP6 binding with plasma proteins within the fibrin meshwork, allows a sustained release of rhBMP6, and avoids generation of antibodies to rhBMP6, thus providing a permissive environment for endochondral bone differentiation. In our previous work (Grgurevic et al., 2018), we have demonstrated that the use of ABC as a carrier reduced the immune response when compared with the use of bovine collagen, as shown by reduced neutrophil accumulation and myeloperoxidase activity in rat subcutaneous implants. The ALLO particles are added and distributed uniformly across the ABC to provide adequate biomechanical and biocompatible good handling properties.

ABGS containing ABC or ABC/ALLO are produced with defined rheological properties. We observed for the first time that ABC has an unexpected inherent biological property as it overcomes foreign body responses elicited by high Ca/P-containing mineralized matrix (ALLO) at ectopic sites. When implanted alone, ALLO did not form bone at subcutaneous (ectopic) site but instead recruited mononuclear phagocytes which then fused to form multinucleated FBGCs to dissolve the mineralized bone matrix implanted at a nonbony site. On the other hand, ALLO implants when combined with ABC reduced significantly the formation of multinucleated FBGCs and with rhBMP6 dispersed within ABC resulted in formation of endochondral bone. It is likely that ABC coated ALLO surface masked T-cell recognition, thus suppressing foreign body response locally. The dose of rhBMP6 required to induce optimal bone formation are comparable in ABGS with or without ALLO as it binds to ABC more tightly than to ALLO and released locally in times with the resolution of ABC by haemolysis. ABGS induced bone is a dose-dependent with an optimal dose of rhBMP6 at 100 µg/ml ABC and produced a complete fusion between two lumbar transverse processes in rabbit PLF model.
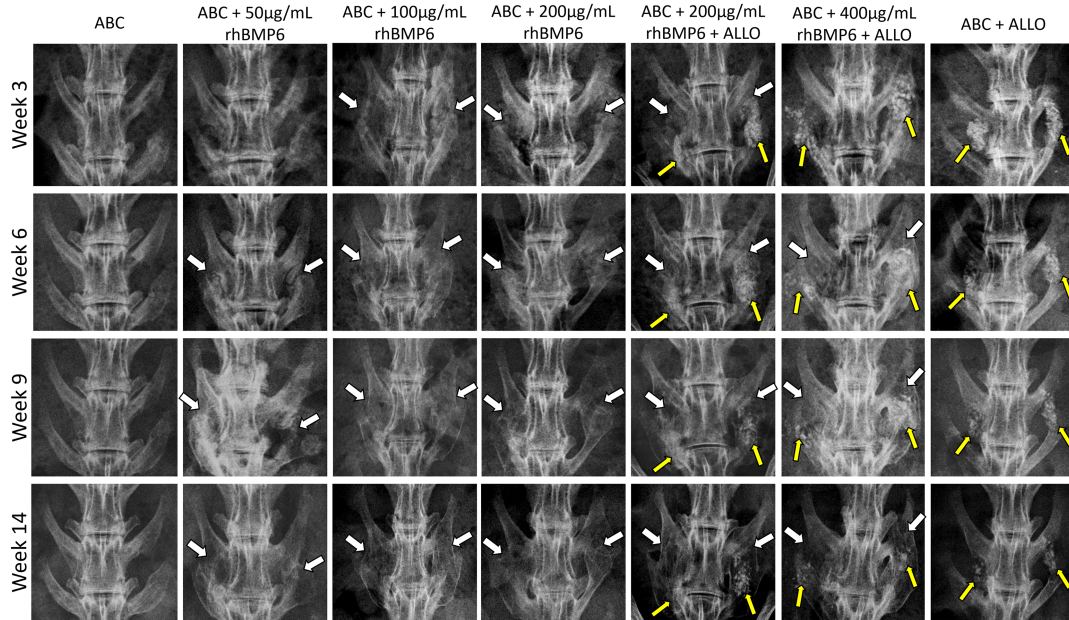
FIGURE 3 Rabbit spinal fusion (L4–L5) imaged in all groups by X-ray at Weeks 3, 6, 9, and 14 during the experiment. White arrows indicate new bone, while yellow arrows indicate allograft particles
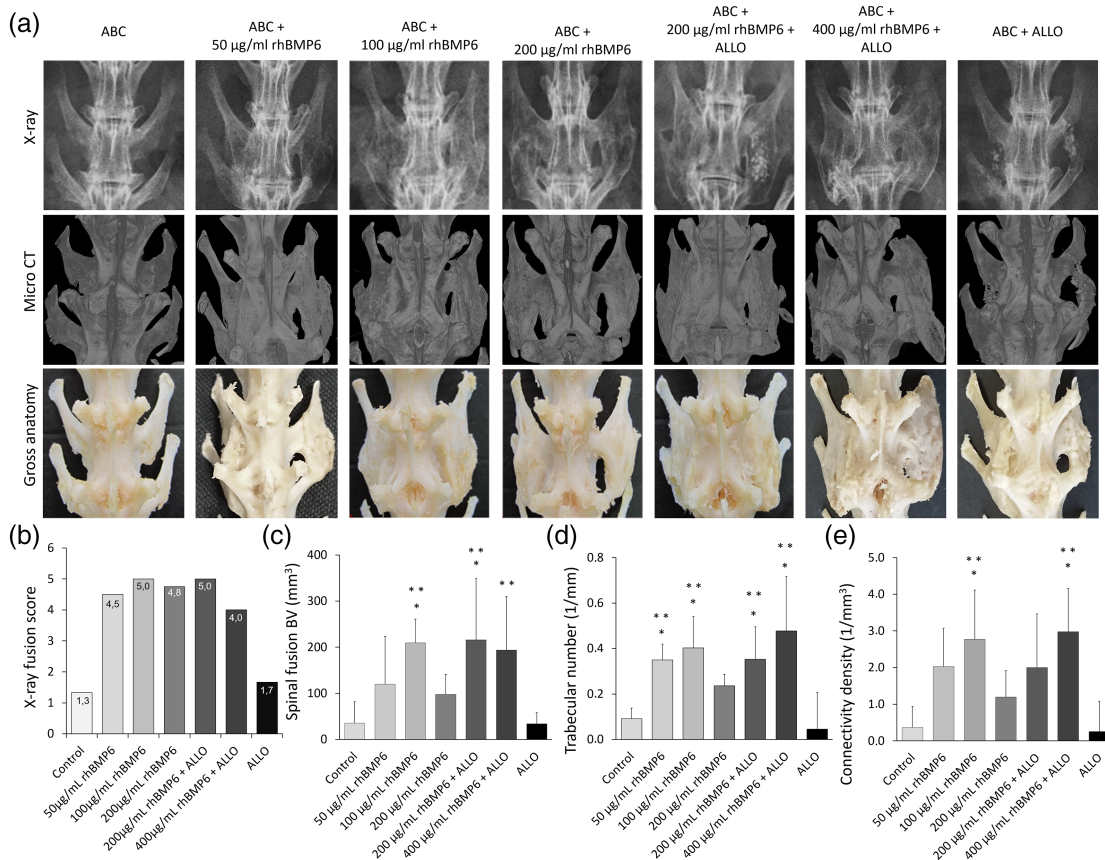

FIGURE 4 (a) Rabbit spinal fusion (L4–L5) after treatment with various doses of rhBMP6 without and with ALLO at 14 weeks after surgery, as visualized by X-ray (top row), micro-CT (middle row), and gross anatomy macerated specimen (bottom row). (b) Spinal fusion X-ray scoring results. (c–e) Spinal fusion morphometric parameters measured from micro-CT images. Results are shown as mean ± SD (n = 4). $^*P$ < .05 versus negative control, $^{**}P$ < .05 versus ABC + ALLO (one-way ANOVA with Tukey post hoc test)
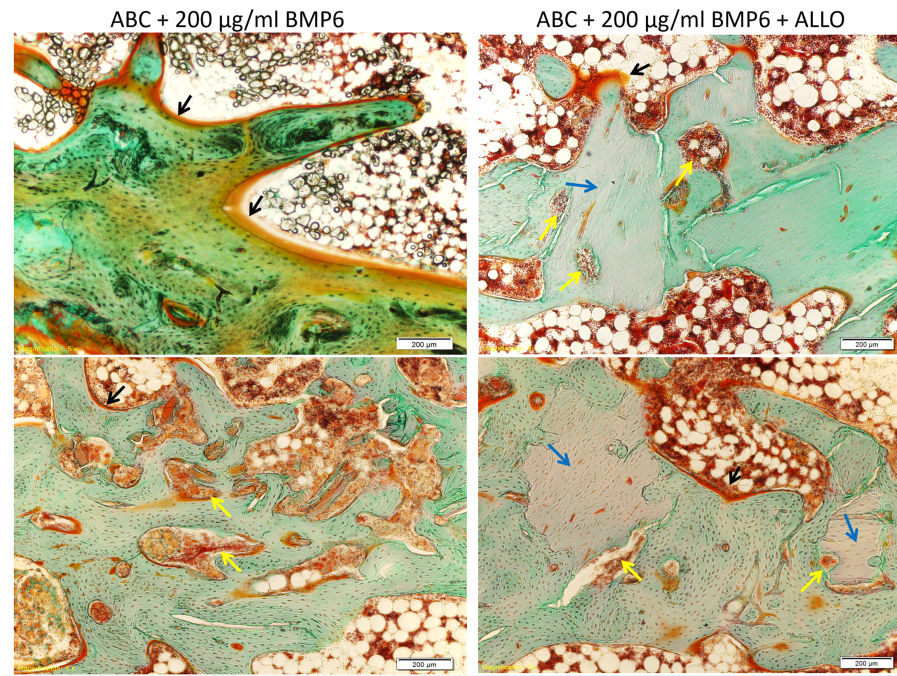
FIGURE 5 Histology of ABGS implants without and with ALLO harvested at 14 weeks after surgery in rabbit PLF model. Left rows represent ABGS implants without ALLO: note newly formed bone containing dense trabecular structure with laid-down osteoid at the surface (black arrows) and pronounced bone remodelling with numerous blood vessels (yellow arrows). Right rows represent ABGS implants with ALLO: The newly formed trabeculae is assimilated with ALLO (blue arrows) via creeping substitution and invaded by new blood vessels (yellow arrows)

The newly formed bone in ABGS containing ALLO was compact and underwent typical bone remodelling as examined by micro-CT analysis and histology, which mimics that of autograft assimilation observed in orthotropic sites (Gupta, Keshav, & Kumar, 2016) via a creeping substitution. ALLO particles provided better structural stability for ABGS, compared with ABGS alone, while permitting the migration of bone forming cells as evidenced by bone formation and ALLO remodelling. We also examined the contribution of decortication versus no decortication of transverse processes prior to placing of the implant, and the results showed that decortication has produced significantly a higher success rate of spinal fusion than keeping the cortices intact.

RhBMP2 (InFUSE) and rhBMP7 (OP-1 Putty) have been shown to induce new bone formation and promote radiographic fusion in PLF animal models (Boden, Moskovitz, Morone, & Toribitake, 1996; Jenis, Wheeler, Parazin, & Connolly, 2002; Martin, Boden, Marone, Marone, & Moskovitz, 1999; Suh et al., 2002; Vukicevic & Sampath, 2017). In contrast to these studies, we show here that rhBMP6 in ABGS induced bone formation at lower doses, and the newly formed bone was restricted to the size and shape of the implants. Although the use of InFUSE (INFUSE Bone Graft product information: Lumbar, 2002) in conjunction with the LT-Cage device has been approved for ALIF procedures in patients with DDD at one level from L2 to S1 (Burkus, Gornet, Dickman, & Zdeblick, 2002), the off-label use for anterior cervical fusion in patients has produced unwanted safety issues that included radiculitis, vertebral body resorption, seroma and/or haematoma formation, uncontrolled heterotopic ossification,

osteolytic erosion, retrograde ejaculation, and concerns of possible cancer risks (Cahill, McCormick, & Levi, 2015; Wong, Kumar, Jatana, Ghiselli, & Wong, 2008), which in part may be attributed to the larger amount of rhBMP2 used in the device (Carragee et al., 2011). Evaluation of rhBMP2 in a porous synthetic slab that is composed of bovine collagen sponge impregnated with ceramic granules of hydroxyl apatite and tricalcium phosphate in PLF clinical studies demonstrated a moderate fusion, but a significant number of patients continued to experience low back pain and leg pain and unwanted adverse events (Dawson, Bae, Burkus, Stambough, & Glassman, 2009; Dimar et al., 2009). RhBMP7 as an implantable bone graft substitute also did not achieve a statistical difference as compared with autograft in a PLF clinical study (Vaccaro et al., 2004; Vaccaro et al., 2008).

Bovine sourced collagens were used as carriers to deliver BMP2 and BMP7. Bovine Achilles tendon derived acid soluble reconstituted Type I collagen mesh as in InFUSE (INFUSE Bone Graft product information: Lumbar, 2002) or as a slab-shaped collagen composite with synthetic ceramics as in Amplify (Executive Summary for P050036 Medtronic's AMPLIFY™ rhBMP-2 Matrix Orthopedic and Rehabilitation Devices Advisory Panel, 2010) were used to deliver rhBMP2. Bovine diaphysis bone derived insoluble Type I collagen as particulate and/or combined with additive carboxymethyl cellulose as injectable putty were used for rhBMP7/OP1 as in OP1-Implants (OP-1 Implant® product information, 2009) and OP1-Putty (OP-1 Putty® product information, 2009). Sterilization of these bovine sourced collagens by chemical methods or gamma radiation for clinical uses added
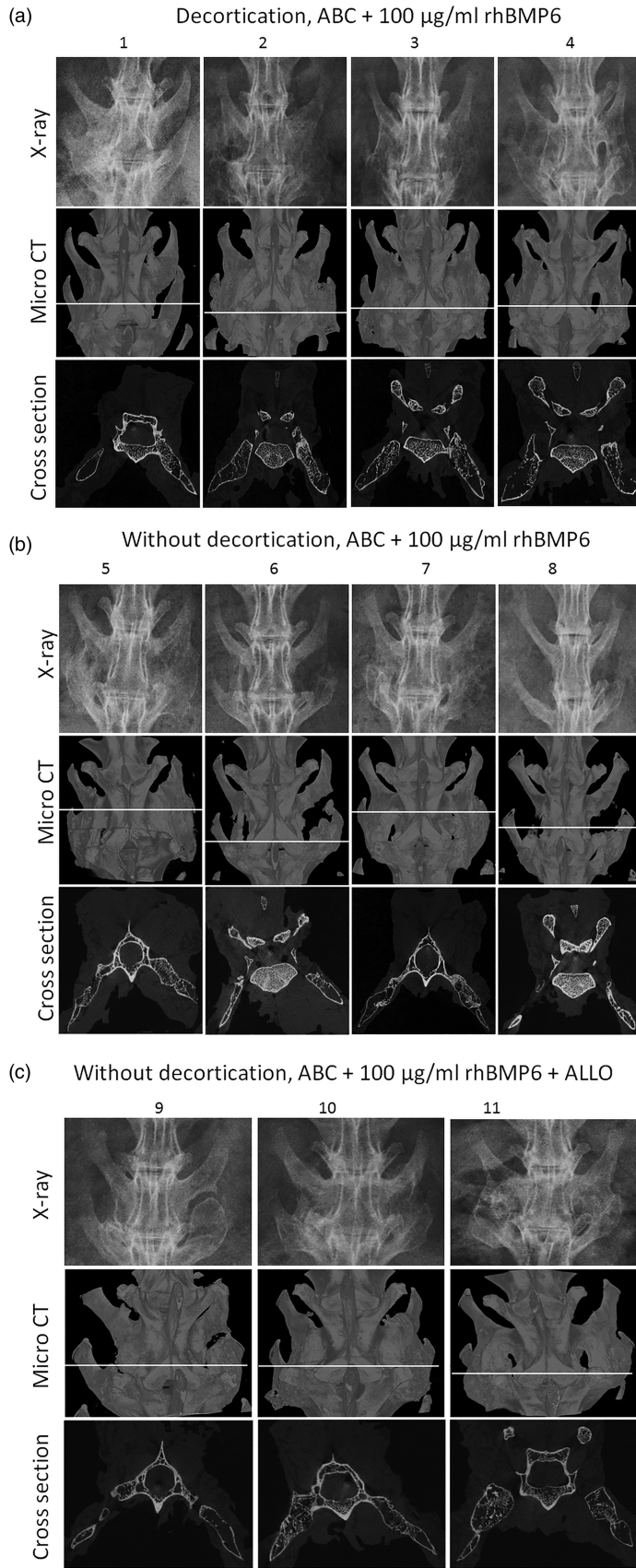
FIGURE 6 Effect of transverse processes decortication on spinal fusion. The same dose of rhBMP6 (100 μg/ml) was used. Rabbit spinal fusion after removal of bone cortex (a), without decortication (b) and in combination with allograft (ALLO) particles (c)

unwanted modifications to collagenous carrier as well (OP-1 Putty® product information, 2009). As such, we present here an ABC to serve as a physiological carrier to deliver rhBMP6 (ABGS) to induce new bone formation and with allograft (bone particle ALLO) as a compression resistant matrix to promote osteogenesis in PLF model and minimize adverse events that may be associated with the use of collagen calcium-ceramics composite scaffold (Govender, Rampersaud, Rickards, & Fehlings, 2002).

ABGS containing ABC and rhBMP6 has been evaluated in a First-in-Human randomized, placebo controlled and double blinded Phase I study in patients with distal radius fracture and in a Phase I/II study in patients with high tibial osteotomy. ABGS with ALLO devices is currently being evaluated in a randomized, double blinded, and controlled Phase II study for posterolateral lumbar interbody fusion against autograft as a comparator.

AUTHOR CONTRIBUTIONS

The authors and their contributions are as follows: L. G., T. K. S., I. E., R. W., S. V., and M. G. (conceptualization); I. E., T. B. N., L. G., S. V., T. K. S., and M. G. (formal analysis); S. V., L. G., I. E., M. P., T. B. N., N. S., M. L., H. C., D. M., R. W., T. K. S., and M. G. (investigation); M. G., M. P., L. G., D. M., I. E., and M. L. (methodology); L. G. and D. M. (project administration); D.M. (supervision); H. C., I. E., N. S., and L. G. (visualization); S. V., T. K. S., T. B. N., L. G., I. E., and M. G. (writing original draft).

CONFLICT OF INTEREST

L. G. and S. V. have an issued patent US8197840 and licensed to Genera Research (GR). R. W. is a consultant for Pfizer, Stryker, Takeda, Depuy Synthes, and Zimmer Biomet; T. K. S. received grants and other from perForm Biologics during the study; S. V. received grants and other from (GR) and perForm Biologics during the study. M. G. is a consultant for Depuy Synthes, Innomed, and Medtronic and receive royalties from Depuy Synthes and Innomed.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. A-D. Histology of rat subcutaneous implants stained for cartilage and bone markers on day 7. **A-B.** ABC/ALLO/rhBMP6 implants stained for SOX-9, the marker for chondrocytes (arrows). **C.** ABC/ALLO/rhBMP6 implants stained for alkaline phosphatase. **D.** ABC/ALLO/rhBMP6 implants stained for osteocalcin. Arrows indicate osteoprogenitor cells.