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Autologous blood coagulum containing rhBMP6 induces new bone formation to promote anterior lumbar interbody fusion (ALIF) and posterolateral lumbar fusion (PLF) of spine in sheep

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ABSTRACT

In the present study, we evaluated an autologous bone graft substitute (ABGS) composed of recombinant human BMP6 (rhBMP6) dispersed within autologous blood coagulum (ABC) used as a physiological carrier for new bone formation in spine fusion sheep models. The application of ABGS included cervical cage for use in the anterior lumbar interbody fusion (ALIF), while for the posterolateral lumbar fusion (PLF) sheep model allograft devitalized bone particles (ALLO) were applied with and without use of instrumentation. In the ALIF model, ABGS (rhBMP6/ABC/cage) implants fused significantly when placed in between the L4-L5 vertebrae as compared to control (ABC/cage) which appears to have a fibrocartilaginous gap, as examined by histology and micro CT analysis at 16 weeks following surgery. In the PLF model, ABGS implants with or without ALLO showed a complete fusion when placed ectopically in the gutter bilaterally between two decorticated L4-L5 transverse processes at a success rate of 88% without instrumentation and at 80% with instrumentation; however the bone volume was 50% lower in the instrumentation group than without, as examined by histology, radiographs, micro CT analyses and biomechanical testing at 27 weeks following surgery. The newly formed bone was uniform within ABGS implants resulting in a biomechanically competent and histologically qualified fusion with an optimum dose in the range of 100 μg rhBMP6 per mL ABC, while in the implants that contained ALLO, the mineralized bone particles were substituted by the newly formed remodeling bone via creeping substitution. These findings demonstrate for the first time that ABGS (rhBMP6/ABC) without and with ALLO particles induced a robust bone formation with a successful fusion in sheep models of ALIF and PLF, and that autologous blood coagulum (ABC) can serve as a preferred physiological native carrier to induce new bone at low doses of rhBMP6 and to achieve a successful spinal fusion.

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1. Introduction

Autografts and allografts containing autologous bone marrow are routinely employed with or without instrumentation to stimulate osteogenesis and promote spine fusion. They are applied either in the intervertebral disc space, as in anterior lumbar interbody fusion (ALIF) [1] or in an ectopic site between two lumbar transverse processes bilaterally as in the posterolateral lumbar fusion (PLF) [2]. A variety of disorders are treated with a spinal fusion, including, but not limited to, degenerative disc disease (DDD), spondylolisthesis, spinal stenosis, scoliosis, infections, spinal fractures, and various tumors, primarily to treat deformity or relieve the source of back and leg pain [3–7].

Autograft from the patient's Iliac crest bone is a “Gold Standard” for spine fusion surgery as the harvested bone chips have live bone marrow cells and an immunologically compatible extracellular matrix [8–10]. However, the use of autograft presents disadvantages as it requires another incision that may result in post-operative pain, infection and the amount of bone that can be harvested is limited [11–18]. As an alternative to autograft, allograft (cadaver bone from a bone bank), demineralized bone matrix (DBM) [19–22], various calcium-based ceramics in conjunction with patients bone marrow [23–27], and bone morphogenetic proteins (BMPs) with animal derived collagen [28–31] and/or with ceramic composite as scaffolds [31–34] have been developed for clinical use [35–37]. The treatment efficacy of lumbar arthrodesis in DDD is a complex clinical and economic issue for patients and health care providers. The rate of nonunion is around 25–36% for non-instrumented PLF and 10% for single-level ALIF [15]. The addition of instrumentation decreased the nonunion rate to 4–12% for PLF, while the use of cages for spinal fusion contributed to higher fusion rates for ALIF and PLF at 2-year follow up [15].

The ability of BMP to induce new bone at ectopic sites upon reconstitution with an appropriate collagenous scaffold serves as a prototype for tissue engineering where BMP serves as a signal and local site provides responding cells to allow bone differentiation under a permissive vascular environment [36,38,39]. In accord with the principles of tissue engineering, rhBMP2 applied within an absorbable collagen sponge, ACS (InFUSE) has been shown to induce new bone formation and promote spine fusion to treat DDD in skeletally mature patients [40] at one level fusion from L2 to S1 using Titanium LT cages via an ALIF [25] approach. Depending on the size of the LT-CAGE the FDA recommended between 4.2 mg and 12 mg BMP per level [41]. However, the off-label use of rhBMP2/ACS in related interbody fusion procedures (e.g., cervical) has resulted in unwanted safety issues likely due to the high dose employed and the use of bovine collagen as a carrier [19,31,42–44]. The clinical evaluation of rhBMP2 soaked in bovine-sourced collagen and synthetic ceramics (hydroxyapatite and tri-calcium phosphate) composite as a scaffold (AMPLIFY) for the PLF procedure [45] was not approved for use as it resulted in unwanted local and systemic safety issues [31,46–49]. Similarly, bovine bone collagen dispersed with additive carboxyl-methyl cellulose that contained rhBMP7 (OP-1 Putty) [50,51] also resulted in disapproval. Subsequently a rise in off-label BMP applications and the lack of guidelines ensued in spinal fusion procedures with a wide range of BMP doses used (2.5–40 mg BMP per level) [52]. This suggested a need for a physiological native carrier instead of animal derived collagen to avoid foreign-body reactions to high-mineral containing Ca-P based ceramics.

We have recently described autologous blood coagulum (ABC) to serve as a physiological native carrier for rhBMP6 as an autologous bone graft substitute (ABGS), which also might contain compression resistant matrix like allograft. ABGS when implanted at ectopic sites significantly reduced the foreign body giant cells response and induced spinal fusion in rabbits without instrumentation following decortication of transverse processes [53,54]. In the present study, we demonstrate that recombinant human BMP6 (rhBMP6), a BMP with high specific bone forming activity due to a low affinity for Noggin, an abundant endogenous BMP antagonist [55], is preferred in spinal surgery. RhBMP6, when delivered in a low dose with ABC alone or with allograft (ALLO), was capable of inducing new bone formation and achieving spinal fusion in sheep models of ALIF and PLF.

2. Materials and methods

2.1. Sheep

Study protocols were conducted in Female sheep (*Ovis aries*), Merinolaandschaf breed, aged 3 to 4 years, with health certificate and weighing 50–70 kg. The animal facility was registered by Directorate of Veterinary, Reg. No: HR-POK-020. Sheep were acclimated for 3 days after transport to the animal facility and randomly assigned to their respective treatment group. They were housed by standard corrals in conventional climate conditions at the temperature of 16–20 °C, relative humidity of 50–70%, and noise level up to 80 dB. Each corral was identified following animal identification, animal strain, study number, group, dose, number and sex of each animal. A standard sheep diet of oats, processed hey, added salt and fresh water was provided ad libitum. Animal care was in compliance with standard operating procedures of the Croatian Animal facility HR-POK-020 using 3R principle and minimization of the pain suffering during the experiment. The guidelines of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123) have been followed.

2.2. ABGS implant preparation

RhBMP6 used in experiments was prepared as follows: lyophilized rhBMP6 (Genera Research, Zagreb) was dissolved in sterile water to a final concentration of 1 mg/mL.

For the ALIF operation, a blood sample for implant preparation was collected from the jugular vein of the animal in tubes without anticoagulant substance. Full blood in the volume of 1.5 mL was supplemented with 0.025 mL of 15 mM $CaCl_2$ solution and 150 μL of rhBMP6 or sterile water. The solution was mixed and transferred to a sterile 10 mL syringe containing CFRP I/F cervical cage (Bengal, DePuy, USA), and allowed to coagulate at room temperature. A typically prepared ABGS implant is depicted in Fig. 1A.

Prior to PLF surgery, blood samples were collected from sheep jugular veins into tubes without anticoagulant. Full blood in the volume of 8 mL was supplemented with 0.1 mL of 15 mM $CaCl_2$ solution and mixed with rhBMP6, according to dose, and then left at room temperature to coagulate. Allogenic devitalized bone particles (ALLO) of 70–420 μm size were prepared as described [56] and were added at 0.3 g per mL of blood/rhBMP6 mix. The mineralized bone particles are distributed evenly as evidenced by X-ray and micro CT images (see Fig. 4A in the Results).

2.3. Anterior lumbar interbody fusion (ALIF) surgical procedure

Female sheep, aged 3–4 years, weighing 50–60 kg were used to test the efficacy of rhBMP6 in the ALIF procedure after implantation of the CFRP cervical cage filled with placebo ABC or 150 μg rhBMP6 in ABC. Sheep were randomly assigned into two groups: ABC alone (n = 5) and ABGS containing 100 μg/mL rhBMP6 (n = 5). The surgeries were carried out under general anesthesia and performed on all animals by the same surgical team. Upon excision of the intervertebral disc (L4-L5) and rasping the cartilage of the end plate, prepared ABGS containing cage was implanted. Fascia and skin were sutured and disinfected.

Sheep were clinically and radiographically supervised by a veterinarian at four different stages: immediately after surgery and at weeks 7 and 11, and 16. During the experiment no adverse effects in any of the animals were observed. The experiment was terminated 16 weeks post-surgery after sedation and premedication with 5 mg/kg xylapane and 20 mg/kg ketamine i.m. and administration of T61 (0.1 mL/kg) i.v. The

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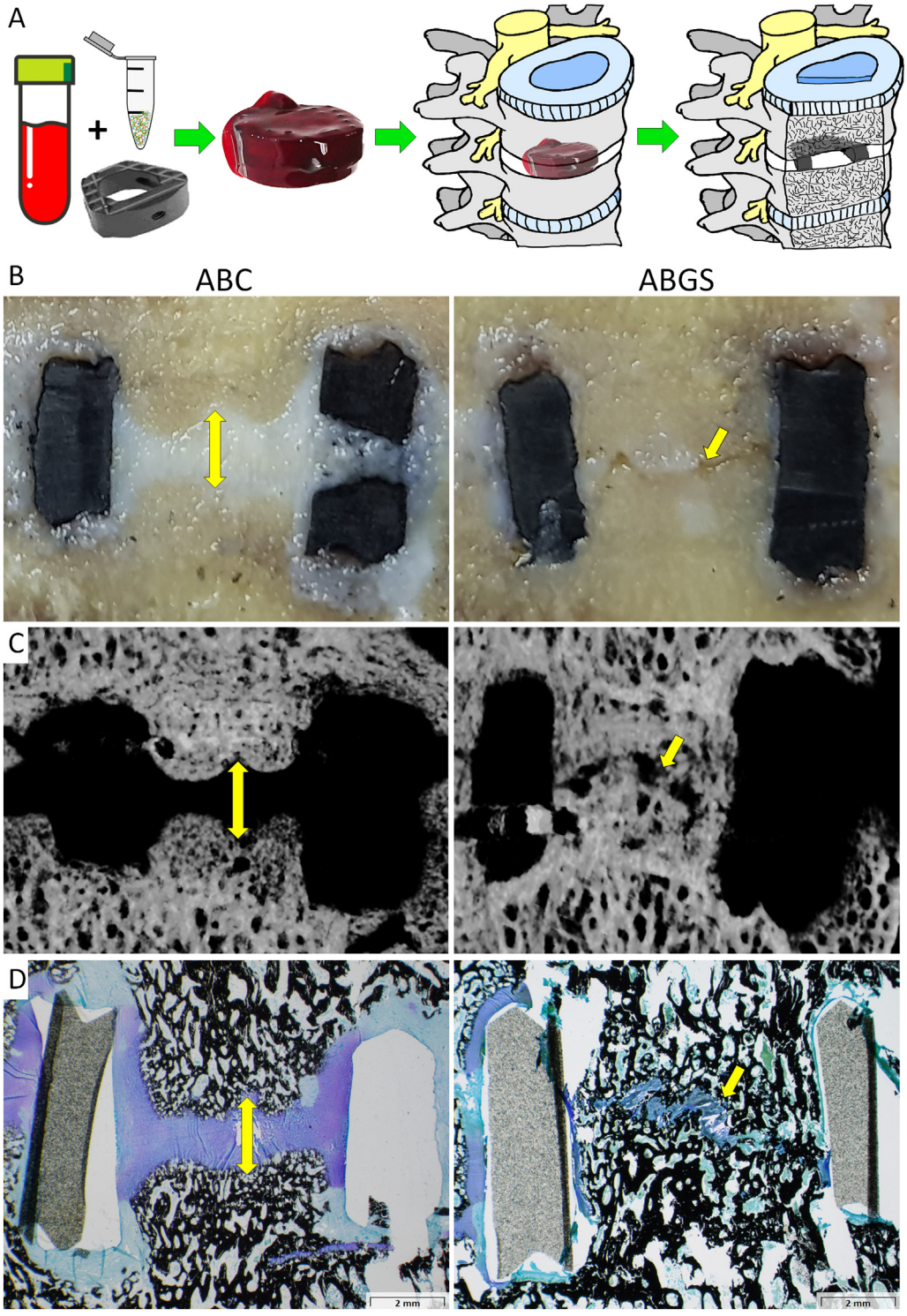

Fig. 1. Preparation and placement of ABGS implants and analyses of harvested implants in sheep ALIF model. A. 1.5 mL of blood was drawn from the jugular vein and mixed with rhBMP6. The cage was immersed into the blood/rhBMP6 mixture and allowed to coagulate for 60 min after which it was implanted in between L4-L5 vertebrae. After 16 weeks, the experiment was terminated, and the lumbar segment of interest was excised and vertically cut through the cage. B. Upon gross examination, the newly formed bone within the cage (black) did not fuse in ABC treated samples (yellow bidirectional arrow), while in ABGS filled cages the fusion was almost complete (unidirectional yellow arrow). C. 3D model of the same specimens scanned by micro CT. D. Histological analysis of the same specimens stained by Von Kossa/MacNeal tetrachrome. Blue area marked by bidirectional yellow arrow indicates a broad unfused area filled with fibrocartilaginous tissue in sheep treated with ABC and almost complete fusion in ABGS treated cage as marked by unidirectional yellow arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spine segments were excised and fixed in 10% formalin for additional analysis by X-ray, micro CT and histology.

2.4. Posterolateral lumbar fusion (PLF) surgical procedure

Two separate PLF experiments were performed in sheep. In the first experiment, 5 female sheep were surgically treated with ABC alone (n = 1) and with ABGS containing 62.5 μg/mL (0.5 mg/implant) rhBMP6 (n = 4). In the second experiment, 12 sheep were administered with ABGS containing 187.5 μg/mL (1.5 mg/implant) rhBMP6 and randomly assigned to three groups: 1) ABGS (n = 3), 2) ABGS plus devitalized sheep ALLO (2 g/implant) (n = 4) and 3) ABGS plus devitalized sheep ALLO (2 g/implant) with instrumentation (n = 5). Blood samples for implant preparation were collected as described and two implants per animal were prepared. The surgeries were carried out under general anesthesia. Spinal fusion was carried out bilaterally in the lumbar region between L4 and L5 vertebrae. Lateral aspect of transverse processes was decorticated until bleeding by a high speed burr (Nouvag AG, High Surg 30, Switzerland) and ABGS implants were placed into the lateral gutter (see Fig. 4A in the Results). Fascia and skin

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were sutured and disinfected. Clinical and radiographical supervision was conducted by a veterinarian immediately after surgery, and at weeks 8 and 27. The experiment was terminated 27 weeks post-surgery after sedation and premedication with 5 mg/kg xylapane and 20 mg/kg ketamine i.m. and i.v. administration of T61 (0.1 mL/kg). Spine segments were excised and fixed in 10% formalin for additional analyses by X-ray, micro CT and histology.

2.5. Methods of evaluation

Radiographical images were taken before the surgery and at noted time points after surgery. X-ray imaging of lumbar spine segments was performed using two standard orthogonal views (lateral and dorso-ventral). Samples were scanned by a Eichermeyer EDR HP (IMD Generators s. r. l., Italy) X-ray machine using the 40 kV and 8 mA settings with all ionization protection protocols respected during the imaging and images were processed using an Agfa CR 30-X (Agfa, Japan). All obtained radiographs from sheep bones were interpreted and scored using the Denver Sheep Fusion Scale for PLF radiographic grading score system [57] by a surgeon and a radiologist blinded to the treatment protocol and postoperative interval. Denver Sheep Fusion Scale for PLF is based on scoring of new fusion from 0 (no bony response) to 5 (bilateral fusion) [57].

Micro CT analysis of sheep lumbar spine spanning from L4 to L7 was done using the SkyScan 1076 (Bruker, Belgium) micro CT device [58]. Spine samples were prepared for scanning by trimming the transverse processes in the ALIF experiments and sawing the spines in half in the PLF experiments. *Ex vivo* lumbar spine samples were scanned at the resolution of 18 μm with concurrent analyses of the site of implantation by CTAn (Bruker, Belgium) software. Morphometric data for bone quantification included bone volume (BV), trabecular thickness (Tb.Th) and separation (Tb.Sp). For ALIF experiments new bone inside the cage and heterotopic ossification outside the cage were manually delineated and analyzed. For PLF experiment the new bone formation and trabecular bone parameters were depicted throughout the whole area of newly formed bone, as previously described [59,60]. Quality of the newly formed bone was compared to the bone in transverse processes (TP) and vertebral body (VB) by delineating a volume of interest (VOI) with 8.5 mm of height and width, and 0.9 mm of depth in each structure with concurrent bone parameters analyses as previously described [53,54,60].

2.6. Histology

Undecalcified histological processing was performed on selected samples following micro CT. The vertebral body on ALIF specimens was cut by oscillating saw in the transversal plane to expose the intervertebral disc space, which permitted central region of the interbody cage/implant and cranial/caudal endplates to avoid orientational metal beads in the cage. This exposed the region of the cage and interior of the new bone for further analysis. PLF specimens were dissected in the transversal plane to expose newly formed bone and adjacent transverse processes. Each specimen was then dehydrated in graded solutions of ethyl alcohol using an automated tissue processing system (ASP300S, Leica Biosystems, USA), and cleared manually with methyl salicylate and xylenes before being polymerized into hardened acrylic resin blocks (MMA). Semi-thin microtome sections were collected in the transversal plane at a thickness of five microns using tungsten-carbide knives (D-profile, Delaware Diamond Knives, USA) and an automated sledge microtome (SM2500, Leica Biosystems, USA). All microtome sections were collected and mounted on custom prepared gelatin coated (Haupt's adhesive) glass microscope slides. Semi-thin microtome sections were deplasticized, hydrated, and stained with hematoxylin and eosin (H&E), modified Goldner's trichrome or Von Kossa/MacNeal's tetrachrome.

2.7. Biomechanical testing

Specimens of newly formed ectopic bone were randomly selected and dissected together with transverse processes from sheep that underwent PLF and biomechanical testing was conducted to determine the maximum force, work to fracture, and elasticity of newly formed bone. Specimens were grouped to those with and without instrumentation and then divided in two subgroups (unilateral and bilateral fusion). The three-point bending test was performed on material testing instrument (TA.HDPlus, Stable Micro Systems, UK) set with a 50 kg load cell. The bone was placed on two supports and force was applied perpendicular to the midpoint. Speed was adjusted at 0.5 mms^{-1}, and force was applied using a single-pronged loading device with flat-tipped wedge [61,62].

2.8. Data management

Values are expressed as mean ± SEM or SD as indicated. Data distribution was checked with the Kolmogorov-Smirnov test and according to the results and the small sample size appropriate non-parametric tests and data description have been used. For statistical comparison of two groups, a two-tailed Student *t*-test was used, while for comparison of more groups two-tailed ANOVA with post hoc Tukey test was used and $P < 0.05$ was considered significant where indicated. Differences between groups regarding force, elasticity and work were analyzed with the Kruskall-Wallis test (all groups together) followed by post-hoc Mann-Whitney *U* test (comparison between each two groups). All data have been shown in Box and Whisker's plots. All *P* values below 0.05 were considered significant. Statistical software IBM SPSS Statistics, version 25.0, have been used in all statistical procedures.

3. Results

3.1. Anterior lumbar interbody fusion (ALIF) study

Upon termination, the lumbar spine segments were excised, fixed, cleaned of soft tissue and vertically cut for further analyses. The gross anatomical structure, presented in Fig. 1B, indicated that implant containing ABC alone did not ossify and fuse fully within the cage (left panel), while the ABGS implant showed a significant area of ossification and fusion of the L4-L5 vertebral bodies (right panel). The bone structure in the cage was further confirmed by micro CT and histology at higher magnification as stained by Von Kossa/MacNeal's tetrachrome (Fig. 1C and D). It was evident that sheep treated with rhBMP6 fused significantly the vertebral bodies as compared to control ABC group.

In the sheep that had received ABGS, newly formed bone was present in and outside the cage, and the new bone fused with both vertebral bodies (Fig. 2A). Micro CT quantitative analyses indicated the fused bone volume was significantly higher in the ABGS treated sheep than in sheep treated with ABC alone. On the other hand, the heterotopic bone observed adjacent to treated vertebral bodies was comparable both in ABC and ABGS groups (Fig. 2B). Histology of the ALIF spine samples is shown in Fig. 3A. In the control ABC group, though bone was formed inside and outside the cage, an area of fibrocartilaginous tissue was present between the cranial and caudal area of newly formed bone and the fusion between vertebral bodies was not achieved. In the ABGS group, areas of newly formed bone both inside and outside the cage were significantly higher and a successful fusion was achieved, except in a few discrete areas in which clusters of dividing chondrocytes and more intensively stained cartilaginous tissue were present (Fig. 3B).

There were no side effects regarding mobility, partial or total paralysis, nerve irritation and/or pain, decreased food intake and weight loss recorded in animals undergoing ALIF procedure. One sheep from the control group died due to pneumonia. The autopsy, performed by a trained veterinary pathologist after 16 weeks, observed no ectopic

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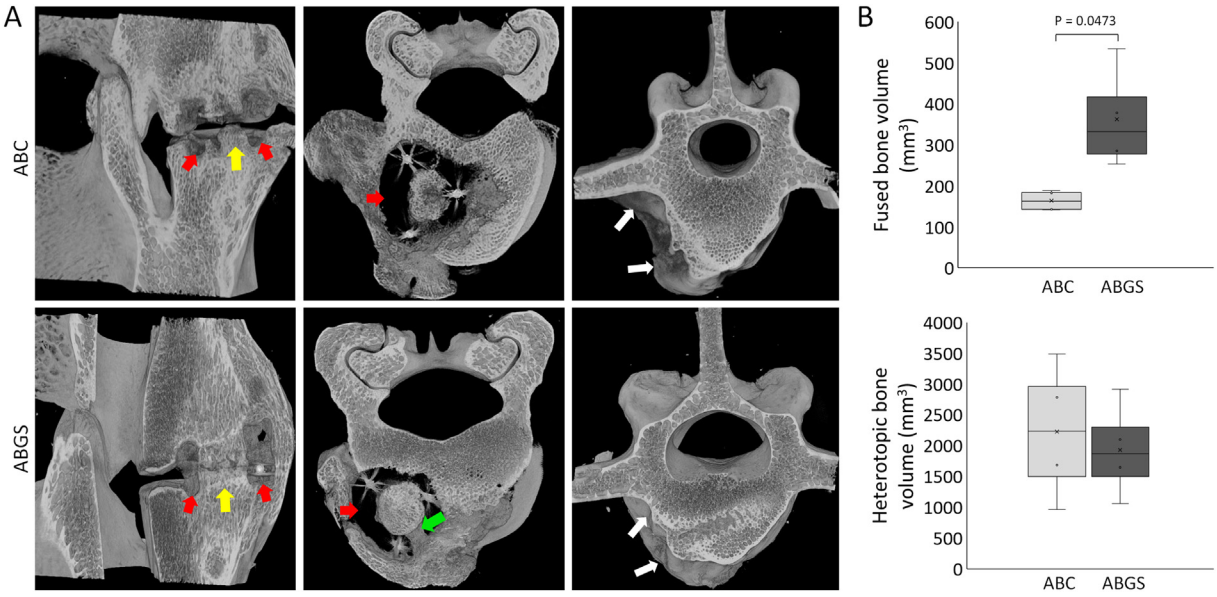

Fig. 2. Micro CT and histological analyses of ALIF sheep specimens. A. In ABC treated sheep, newly formed bone (yellow arrow) within the cage (red arrows) was not fused, while in the sheep treated with ABGS the bone within the cage (red arrows) is almost completely fused (yellow arrow). In the ABGS treated sheep (middle panel), additional bone ingrowth through the cage (red arrows) openings (green arrow) was observed. In the right panels, heterotopic ossification (white arrows) was observed both in ABC and ABGS specimens. B. Micro CT morphometric analyses show significant increase of bone volume within the cage of ABGS treated sheep, while no difference in heterotopic ossification was observed between groups. Data were presented as box-and-whisker plots with mean, median and all values ($n = 6$) and analyzed by the Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ossifications, edema, swelling, tumors or any other visible gross changes. During the entirety of the study, no incidence of infection was noted. The use of ABGS resulted in significant fusion of two adjacent lumbar vertebrae, as compared to incomplete fusion in control animals.

3.2. Posterolateral lumbar fusion (PLF) study

ABGS at a dose of rhBMP6 62.5 μg/mL ABC (0.5 mg/implant) or rhBMP6 187.5 μg/mL ABC (1.5 mg/implant) induced new bone formation and achieved a complete fusion when harvested at 27-weeks post implantation. ABC alone implants have failed to induce bone and achieve spine fusion (data not shown). ABGS with ALLO at 187.5 μg/mL rhBMP6 (1.5 mg/implant) implanted with and without instrumentation also induced new bone formation. Success rate was 88% without instrumentation, and 80% with instrumentation, as determined using the Denver Sheep Fusion Scale for PLF [57]. Fig. 4B shows the images of radiographs (top panel) and micro CT (bottom panel). Morphometric parameters, bone volume (BV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) of the fused bone as determined from micro CT analyses were comparable between the two dose groups (Fig. 4C) of ABGS without ALLO. ABGS/ALLO implants at 187.5 μg/mL showed significantly higher bone volume when implanted without instrumentation as compared to ABGS without ALLO. However, in ABGS with ALLO implants and instrumentation the bone volume was reduced significantly (50%) but was comparable to ABGS without ALLO. Similar to ALIF animals, no side effects were recorded in sheep undergoing PLF, including any visible morphological changes.

Fig. 5A left shows the gross examination of new bone fused between two transverse processes bilaterally. Fig. 5A middle and right panels show the fusion sites at high magnification. The osseointegration is evident and indistinguishable without any demarcation at the juncture of the new bone with native bone transverse processes. Micro CT analysis further confirmed the quality of osseointegration with the transverse process as observed by gross anatomy examination (Fig. 5B left). At higher magnification (Fig. 5B middle and right panels), it was evident that the trabeculae of new bone were merging and connecting with the trabeculae of native transverse bone processes. Bone volume and trabecular separation as assessed by quantitative micro CT analysis showed the new bone was stronger than the native bone of transverse process or the vertebral body (Fig. 5C).

Successful osseointegration of the newly formed bone with the transverse processes was further confirmed on histological sections (Fig. 5D). Bone marrow of new bone and bone in the transverse processes differed because the predominant cells in the bone marrow of new bone were adipocytes, while hematopoietic cells dominated the bone marrow of transverse processes (Fig. 5D). The border between them was sharp. Based on cell population present in the bone marrow, it was possible to visualize the fusion line between the new bone and the bone of the transverse processes. Implanted ALLO was completely resorbed in implants and there was no pronounced histological difference among the experimental groups.

3.3. Biomechanical testing

To examine the biomechanical strength, specimens of fused bones from the PLF study were randomly selected and the three-point bending test was performed to determine the maximum force, work-to-break and elasticity. Specimens were selected to those with and without instrumentation and then divided into unilateral and bilateral fusion samples. Unilateral failure in two animals was associated with a broken ABC coagulum in the middle part which occurred during inappropriate manipulation prior to implantation, but the surgical team's decision was to proceed with testing the performance of such implants. The bone volume was 50% higher in the contralateral implants of those sheep than in sheep with the symmetrical bilateral implants. The bone volume was adjusted due to mechanical loading through the axial spine weight bearing transfer. The mechanical strength of the newly formed bone without instrumentation was higher than the native transverse processes bone. However, with instrumentation the strength of new bone was considerably lower. These biomechanical parameters were supported by the micro CT findings (Fig. 6A–B). When spinal fusion was achieved unilaterally, both maximum force and work-to-break of the newly formed bone were higher when compared to bilateral specimens (Fig. 6D–E).

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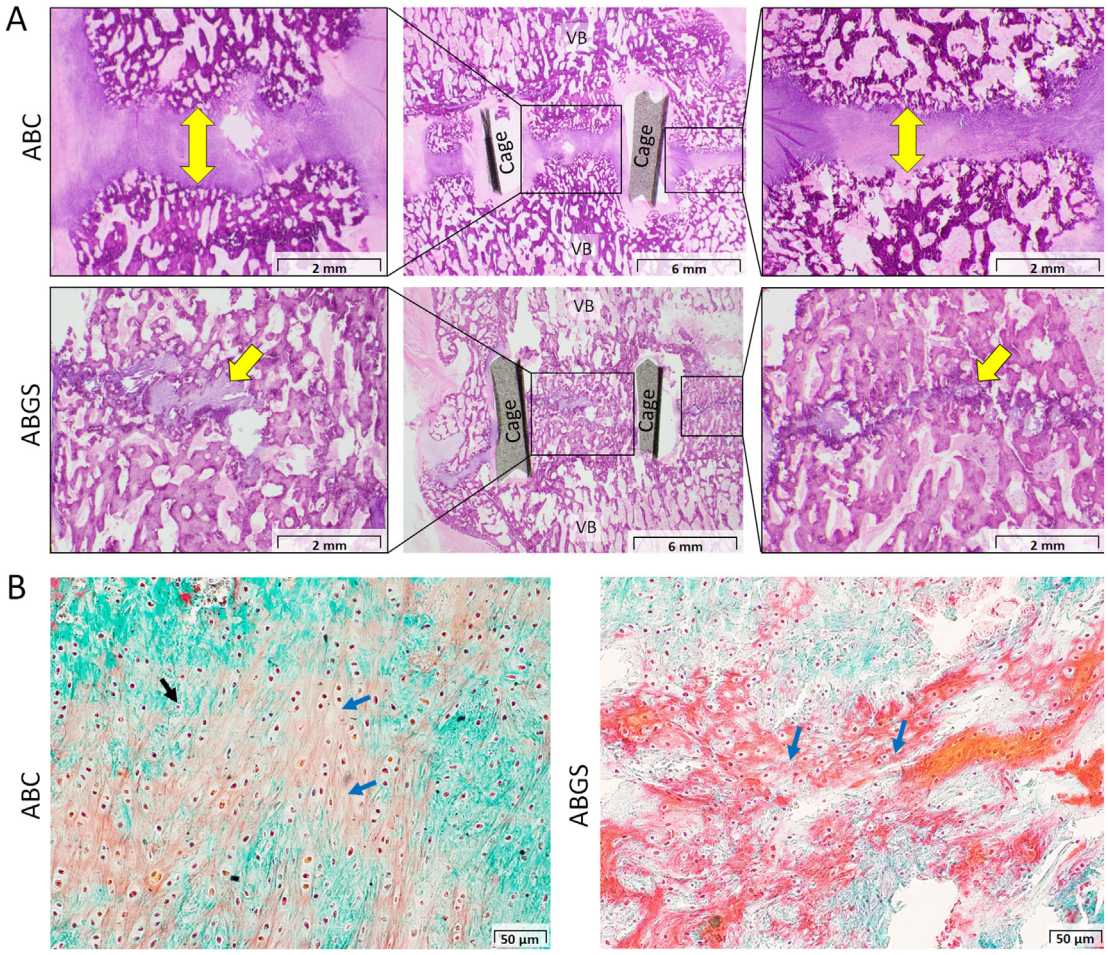

Fig. 3. A. Histology sections of ABC and ABGS implants stained by Hematoxylin/Eosin. Unfused area, both inside (left panels) and outside (right panels) of the cage as shown by bidirectional yellow arrows was present in ABC treated sheep. In ABGS treated sheep the newly formed bone was almost completely fused (unidirectional yellow arrows). VB stands for vertebral body, while CAGE stands for perpendicularly cut cage through the spine segment. B. Magnification of histology sections of ABC and ABGS implants from Fig. 3A stained by Goldner's trichrome. An abundant fibrocartilaginous area was present in the ABC group, while clusters of dividing chondrocytes with more intensively stained cartilaginous tissue were present in the narrow areas of progressing ossification in ABGS treated specimens. Black arrow indicates fibrous tissue; blue arrows indicate cartilage composed of chondrocytes embedded in cartilaginous extracellular matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

We demonstrated for the first time that autologous bone graft substitute (ABGS) containing recombinant human BMP6 (rhBMP6) dispersed within autologous blood coagulum (ABC) achieved a successful spinal fusion when applied as an implant inside and surrounding the implanted cage between L4 and L5 vertebra as in anterior lumbar interbody fusion (ALIF) procedure. In addition, when placed as a cylinder shaped implant posteriorly, ABGS resulted in a successful fusion at ectopic bilateral sites between L4-L5 transverse processes as in posterolateral lumbar fusion (PLF) procedure. In parallel, the sheep that received ABC alone failed to achieve spinal fusion in both ALIF and PLF models as examined at 16 weeks and 27 weeks, respectively, following the surgery. The newly formed bone and the quality of lumbar fusion was qualified by radiographs and histology and quantified by micro CT and biomechanical parameters [63]. In the PLF model, evaluation of ABGS combined with sheep ALLO particulates used as compression resistant matrix improved biocompatibility as well as handling properties and resulted in fusion comparable to ABGS without ALLO. The use of instrumentation to stabilize the lumbar segments, as it is used routinely in the PLF procedures, also resulted in fusion but the amount of bone volume as quantified by micro CT was reduced when compared to the procedure without instrumentation. The stiffness and lack of motion with instrumentation may have contributed to this reduced bone volume [64]. It needs to be noted that we have not yet evaluated the ABGS without ALLO with instrumentation. It remains to be examined whether the mechanical rigidity has a negative influence on bone formation particularly in four-legged animals.

BMP, as an injectable drug, is not efficient for local bone formation, so originally it was delivered with an appropriate collagenous carrier to stimulate osteogenesis in preclinical studies [39]. Although autologous and/or allogenic collagenous matrices are preferred to minimize immune insults in humans, the clinically approved rhBMP2 or rhBMP7/OP-1 based bone graft substitute have employed animal (bovine) derived collagens. For spine PLF indications, these collagen scaffolds are combined with highly mineralized Ca-P and/or carboxymethyl cellulose to achieve acceptable handling properties. These composite implants invariably result in immunological and foreign-body responses at the local implant sites. To overcome this unwanted biology, high doses of BMPs have been employed [19,31]. As BMP2 is known to bind avidly to Noggin, a BMP antagonist predominant in the bone, and to exhibit a weak affinity to collagen/mineral composites, high doses of BMP2 (12–40 mg/site) have been employed in preclinical and clinical studies. BMP2 dissociates at the implant sites resulting in unwanted local and systemic safety issues and in inconsistent lumbar fusion in humans.

Autologous blood coagulum (ABC) in ABGS serves as a native

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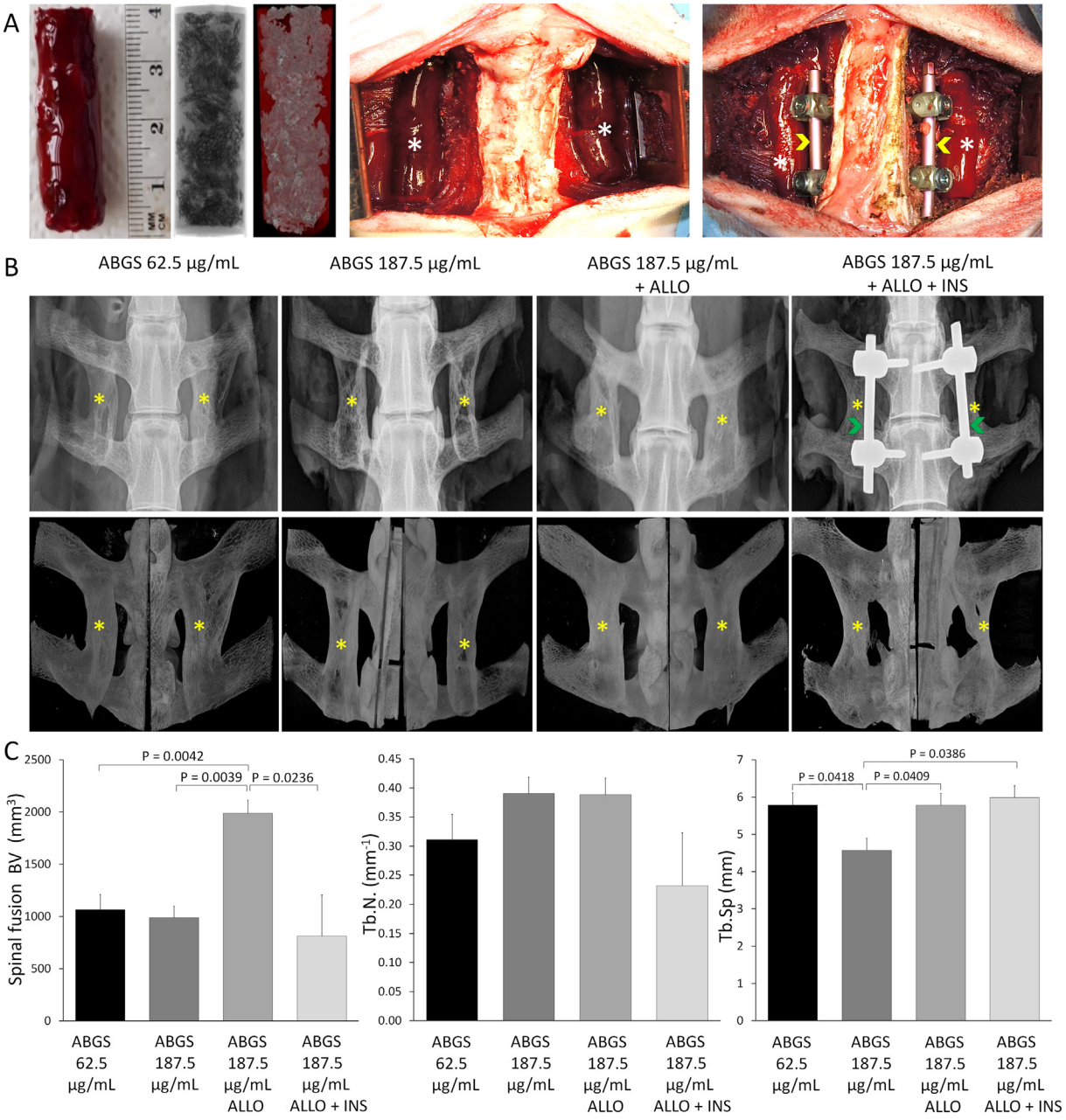

Fig. 4. Preparation and placement of ABGS implants and analyses of harvested implants at 27 weeks after surgery in sheep PLF model. A. ABGS implants with ALLO particles were produced as described in Materials and Methods; a representative implant shows that allograft bone particles are distributed uniformly within ABGS implants as shown by gross, X-ray and micro CT images (left panel). A photograph of ABGS/ALLO implant (white asterisk) placement in the gutter between two transverse processes is shown without instrumentation (middle panel) and with interpeduncular screws and rods (right panel, yellow arrowhead). B. Radiographs (upper panels) and micro CT (lower panels) analyses from representative ABGS implants are shown. Note that ABGS implants induced new bone (yellow asterisks) which achieved a complete fusion between two transverse processes both at a dose of 62.5 µg and 187.5 µg of rhBMP6/mL of ABC without ALLO, or with ALLO respectively. ABGS implants also induced new bone formation and lumbar fusion at a dose of 187.5 µg rhBMP6/mL of ABC with ALLO and using instrumentation (the utmost right panels). C. Micro CT morphometric analysis of bone volume, trabecular number and trabecular separation of the treatment regimens as indicated. Results are shown as mean ± SD ($n = 6$). * $P < 0.05$ vs. 187.5 µg/mL rhBMP6 + ALLO + INS, ** $P < 0.05$ vs. 62.5 µg/mL (one-way ANOVA with Tukey post-hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

physiological carrier for BMP6 and its advantages, as compared to commercially used rhBMP2 on bovine collagen molecule are: 1) provides circulating blood-borne osteoprogenitors, 2) promotes rhBMP6 binding with plasma proteins tightly within the fibrin mesh-work and slow release of the intact protein, 3) decreases inflammation and when used with highly mineralized devitalized ALLO suppresses the formation of multinucleated giant cells [53], 4) reduces immune responses and avoids generation of antibodies to rhBMP6 in rabbits [54] and humans [65], and 5) provides a permissive environment for bone differentiation by its buffering capacity. Additionally, BMP6 binds reversibly to Noggin, a natural BMP antagonist present in bone, has affinity for most of the type I and II BMP receptors and has a high specific alkaline phosphatase activity in osteoblastic cell cultures as compared to BMP2 or BMP7 [55,66,67].

ABGS examined at 100 µg rhBMP6/mL ABC in ALIF and at 62.5 µg rhBMP6/mL ABC and 187.5 µg rhBMP6/mL ABC in PLF resulted in successful fusion. It appears 100 µg rhBMP6/mL ABC is an optimal dose as there is no significant difference in the quantity of bone formed and

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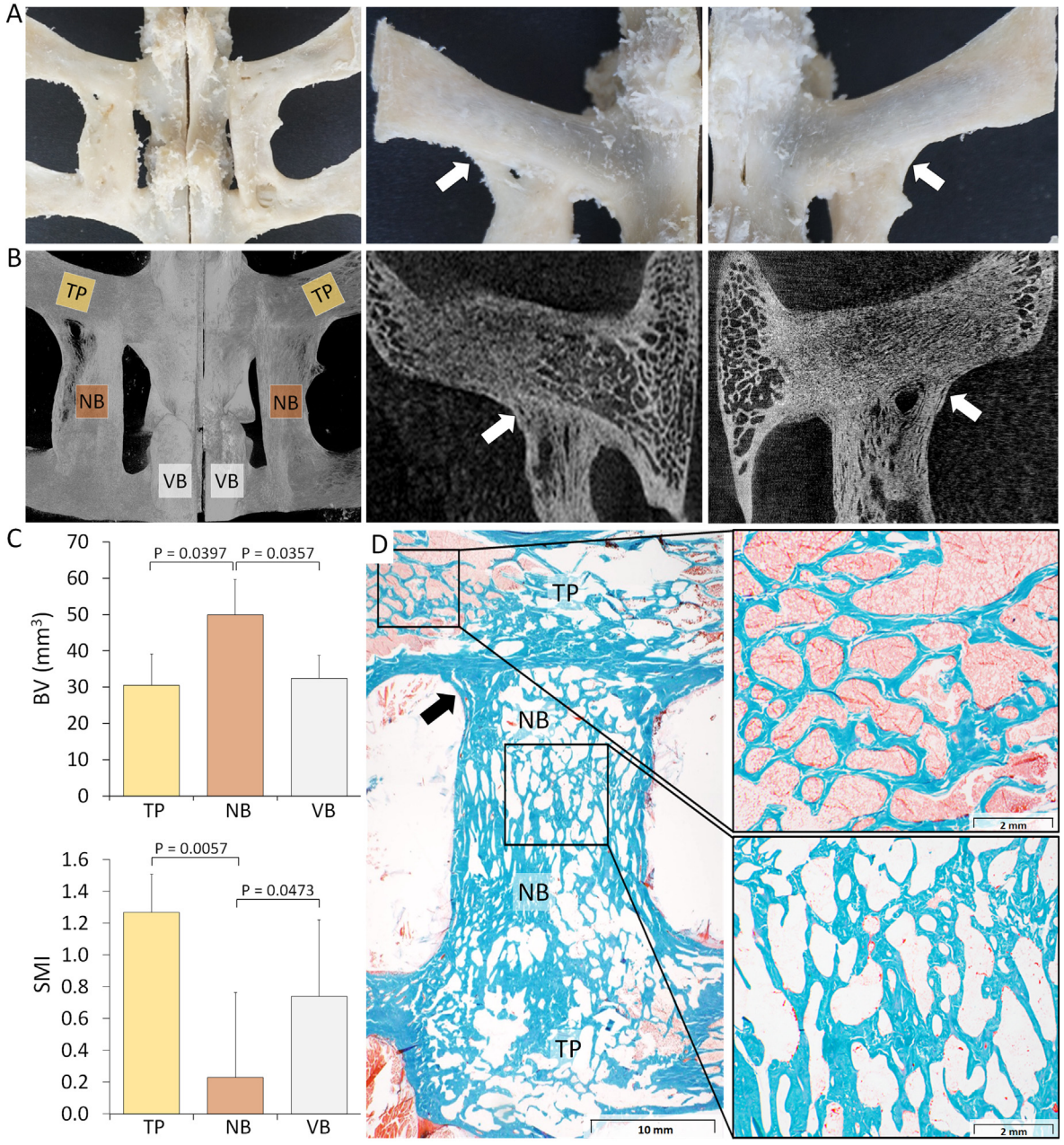

Fig. 5. Photographs of fused bones between two transverse processes from a representative sheep treated with ABGS/ALLO containing 187.5 μg/mL rhBMP6 bilaterally in PLF model. A specimen was macerated from soft tissues. A. Gross-anatomical structure showing the complete fusion between transverse processes and newly formed bone is shown bilaterally (Left panel). Higher magnification shows the integration of fused bone (white arrows) at the juncture of transverse processes (middle and right panels). B. Micro CT confirmation of the fully fused newly formed bone between the transverse processes at low magnification (left panel). Representative sites for morphometric analysis by micro CT are shown by TP (transverse processes), NB (new bone) and VB (vertebral body). The juncture of fused bone with transverse processes at higher magnification was shown (middle and right panels). C. Morphometric analyses of bone volume (BV) and structure mode index (SMI) of newly formed bone were compared to transverse processes and the vertebral body was analyzed by one-way ANOVA with Tukey post-hoc test. *$P < 0.05$ vs. vertebral body, **$P < 0.05$ vs. transverse process. D. Histology photograph of the new bone (NB) incorporated (black arrow) into the transverse processes (TP) on undecalcified bone sections 4.5 cm long and 5 μm thick with Goldner Trichrome staining at magnification indicated with the scale bar (10 mm). In the right panels, TP and NB are magnified (10×) to indicate different bone marrow contents.

the quality of fusion with a higher dose as seen in the PLF model. Studies using rhBMP2 soaked in bovine tendon derived absorbable collagen sponge (ACS) and applied within a cortical dowel allograft or a threaded titanium interbody cage were shown to promote anterior interbody L7-S1 lumbar fusion at a dose of 1.5 mg/mL concentration in a nonhuman primate and in sheep ALIF models [57,68,69]. The addition of HA/TCP synthetic ceramic granules or allograft bone chips with absorbable collagen sponge (ACS) also was shown to promote fusion with rhBMP2 in the posterolateral lumbar fusion preclinical models albeit at high doses [34].

RhBMP2/ACS (INFUSE) has been approved as an autograft substitute for treating DDD at one level vertebra-disc-vertebra from L2 to S1 using an anterior approach in skeletally mature patients. Each device contains 12 mg of rhBMP2 (in total) and includes a sheet of collagen soaked with 6 mg and filled separately in 2-LT cages that are inserted into the intervertebral disc space. The efficacy of the device has also been the subject of a number of studies [22,25]. However, the off-label use to promote spinal fusion with posterior approaches has produced

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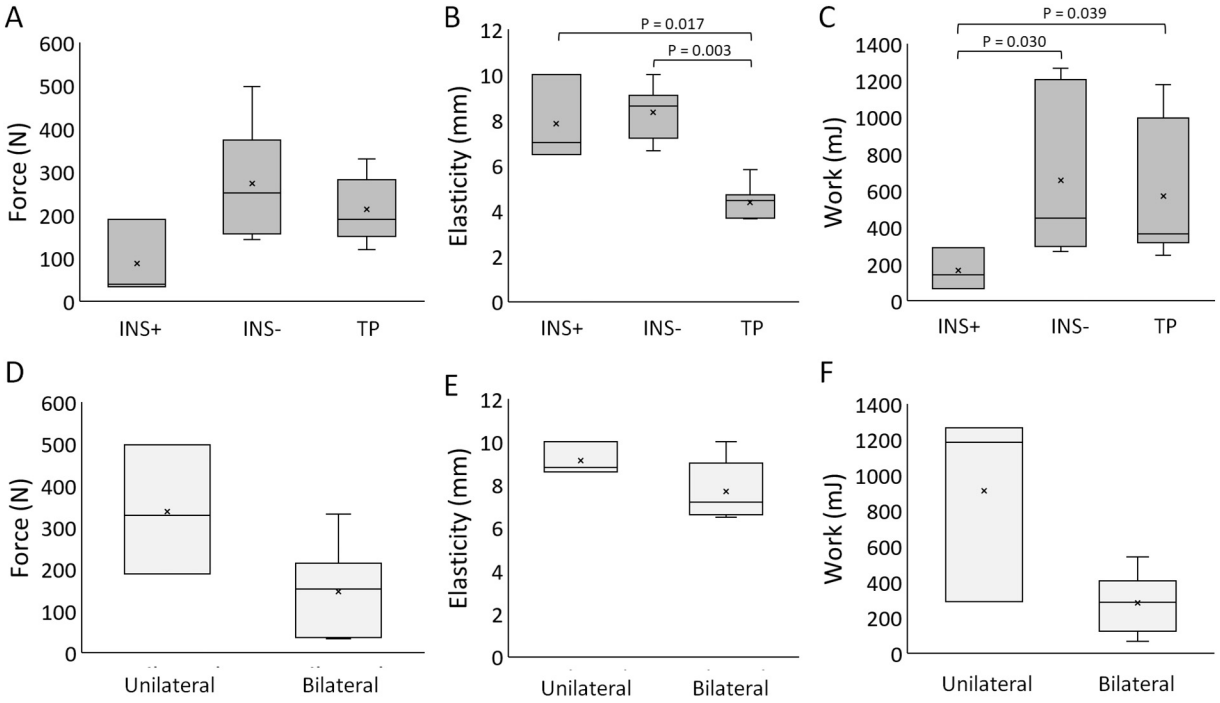

Fig. 6. Biomechanical analysis of the samples from PLF sheep treatment groups. A–C. Comparison of dissected newly formed bone samples obtained from INS + and INS- groups with the transverse processes alone (TP). D–F. Fused bone obtained from bilateral implants as compared with unilateral implants. Force, work and elasticity have been individually determined and analyzed. Results are shown as box and whisker plots (n = 3–6). * $P < 0.05$ INS- vs INS +: and bilateral vs unilateral (Kruskal Wallis test with post-hoc Mann Whitney U test).

unwanted safety concerns [25,46]. RhBMP7/Collagen/CM-Cellulose (OP-1 Putty) has also not been approved for PLF; however, both rhBMP2 (INFUSE) and rhBMP7 (OP-1 Putty) have been allowed for humanitarian device exemption (HDE) use in PLF where the autograft is not feasible.

The dose that we found in the current ALIF and PLF studies in sheep is comparable to the reported dose in our recently published rabbit PLF study [54] and in our rabbit ulna study [53], suggesting the optimal dose of 100 μg/mL ABC is translatable from small (rabbits) to large animals (sheep). These observations supported our clinical study design to evaluate ABGS/ALLO with instrumentation in posterolateral lumbar interbody fusion (PLIF) in humans (https://osteoprospine.eu). The prepared cylinder-shaped ABGS/ALLO implants exhibit a uniform distribution of ALLO particles across the ABC providing biocompatibility, good handling properties, and a sustainable release of rhBMP6 over 7–10 days as examined *in vitro* [54]. This rhBMP6 release is likely to follow in accordance with the dissolution of blood coagulum *in vivo*. A randomized, double-blinded controlled Phase II study on posterolateral lumbar interbody fusion (PLIF) utilizing ABGS with human devitalized allograft particulates and is being conducted at the Department of Orthopedics and Traumatology, AKH University Hospital in Vienna.

5. Conclusions

We provide evidence that ABGS in which ABC served as a native physiological carrier for a small dose of BMP6 in ALIF and PLF sheep models. In the ALIF model, implanted ABGS containing rhBMP6 in ABC/cervical human cage fused significantly when placed in between the L4-L5 vertebrae as compared to control implants for a 16-week period. In the PLF model, ABGS implants either with or without ALLO showed a complete fusion at a success rate of 88% without instrumentation and at 80% with instrumentation, as examined by histology, radiographs, micro CT analyses and biomechanical testing at 27 weeks following surgery. In the implants that contained ALLO, the mineralized bone particles were substituted by the newly formed remodeling bone via creeping substitution. We believe the novel ABGS will provide an acceptable approach to treat spine disorders in patients.

CRediT authorship contribution statement

Lovorka Grgurevic: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Igor Erjavec:** Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Munish Gupta:** Conceptualization, Methodology, Validation, Investigation. **Marko Pecin:** Methodology, Validation, Investigation. **Tatjana Bordukalo-Niksic:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing. **Nikola Stokovic:** Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Drazen Vnuk:** Methodology, Validation, Formal analysis, Investigation. **Vladimir Farkas:** Methodology, Validation, Investigation. **Hrvoje Capak:** Methodology, Validation, Investigation. **Milan Milosevic:** Methodology, Validation, Formal analysis, Investigation. **Jadranka Bubic Spoljar:** Methodology, Validation, Investigation. **Mihaela Peric:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Supervision, Project administration. **Mirta Vuckovic:** Methodology, Validation, Investigation. **Drazen Maticic:** Conceptualization, Methodology, Validation, Investigation, Supervision. **Reinhard Windhager:** Methodology, Validation, Formal analysis, Investigation. **Hermann Oppermann:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision. **T. Kuber Sampath:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision. **Slobodan**

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Vukicevic: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

LG, HO and SV have an issued patent US8197840 licensed to Genera Research (GR). HO received grants and other from GR during the study, RW is a consultant for Pfizer, Stryker, Takeda, Depuy Synthes and Zimmer Biomet, TKS received grants and other from perForm Biologics during the study. MG is a consultant for Depuy Synthes, Innomed and Medtronic and receive royalties from Depuy Synthes and Innomed.

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