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A novel autologous bone graft substitute comprised of rhBMP6 blood coagulum as carrier tested in a randomized and controlled Phase I trial in patients with distal radial fractures

Dragan Durdevic[a,1], Tomislav Vlahovic[a,1], Sanja Pehar[b], Dina Miklic[a], Hermann Oppermann[c], Tatjana Bordukalo-Niksic[b], Ismet Gavrankapetanovic[d,2], Mehmed Jamakosmanovic[d], Milan Milosevic[e], Snjezana Martinovic[f], T. Kuber Sampath[g], Mihaela Peric[h], Lovorka Grgurevic[b], Slobodan Vukicevic[b,*]

[a] Clinical Hospital Center "Sisters of Mercy", Clinic of Traumatology, Draskoviceva 19, 10000 Zagreb, Croatia
[b] Laboratory for Mineralized Tissues, Centre for Translational and Clinical Research, University of Zagreb School of Medicine, Salata 11, 10000 Zagreb, Croatia
[c] Genera Research, Svetonedeljska 2, Kalinovica, 10436, Rakov Potok, Croatia
[d] University Clinical Center Sarajevo, Clinic of Orthopedics and Traumatology, Bolnicka 25, 71000 Sarajevo, Bosnia and Herzegovina
[e] School of Public Health "Andrija Stampar", University of Zagreb School of Medicine, Rockefellerova 4, 10000 Zagreb, Croatia
[f] SmartMedico, Zupanova 5, 10000 Zagreb, Croatia
[g] perFormBiologics Inc., Holliston, MA 01746, USA
[h] Department for Intracellular Communication, Centre for Translational and Clinical Research, University of Zagreb School of Medicine, Salata 2, 10000 Zagreb, Croatia

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ABSTRACT

Bone morphogenetic proteins (BMPs) are known to induce new bone formation in vivo but treating trabecular bone defects with a BMP based therapeutic remains controversial. Here, we evaluated the safety and efficacy of a novel Autologous Bone Graft Substitute (ABGS) comprised of recombinant human BMP6 (rhBMP6) dispersed within an autologous blood coagulum (ABC) as a physiological natural carrier in patients with a closed distal radial fracture (DRF). We enrolled 32 patients in a randomized, standard of care (SoC) and placebo (PBO) controlled, double-blinded Phase I First in Human (FiH) clinical trial. ABGS was prepared from peripheral blood as 250 μg rhBMP6/mL ABC or PBO (1 mL ABC containing excipients only) and was administered dorsally via a syringe injection into the fracture site following closed fracture fixation with 3 Kirschner wires. Patients carried an immobilization for 5 weeks and were followed-up for 0 to 26 weeks by clinical examination, safety, serial radiographic analyses and CT. During the 13 weeks follow-up and at 26 weeks post study there were no serious adverse reactions recorded. The results showed that there were no detectable anti-rhBMP6 antibodies in the blood of any of the 32 patients at 13- and 26-weeks following treatment. Pharmacokinetic analyses of plasma from patients treated with ABGS showed no detectable rhBMP6 at any time point within the first 24 h following administration. The CT image and radiographic analyses score from patients treated with AGBS showed significantly accelerated bone healing as compared to PBO and SoC at 5 and 9 weeks (with high effect sizes and $P = 0.027$), while at week 13 all patients had similar healing outcomes. In conclusion, we show that intraosseous administration of ABGS (250 μg rhBMP6/mL ABC) into the distal radial fracture site demonstrated a good tolerability with no serious adverse reactions as well as early accelerated trabecular bone healing as compared to control PBO and SoC patients.

* Corresponding author at: Laboratory for Mineralized Tissues, Center for Translational and Clinical Research, University of Zagreb School of Medicine, Salata 11, 10000 Zagreb, Croatia.
E-mail address: slobodan.vukicevic@mef.hr (S. Vukicevic).
[1] Equal contribution.
[2] Present address: Private Orthopaedic Clinic "prof. dr. Ismet Gavrankapetanovic", Terezije 38, 71000 Sarajevo, Bosnia and Herzegovina.

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1. Introduction

Non-union bone fractures lead to a great and often irreversible loss of quality of life and are associated with an increase in mortality. It is not only the fracture itself that has these detrimental effects, but the associated events and complications that appear during the long time needed for a fracture to heal. Therefore, even a moderate reduction in the time to regain the use of the fractured limbs would be of benefit. Every year 8 million bone fractures occur in the U.S. and another 30 million fractures are registered elsewhere [1,2]. As the population ages, it is predicted that in the EU more than 12 million bone fractures will occur yearly by 2050. Approximately 2.5 million bone grafting operations are performed annually in EU and US [3]. It is estimated that approximately 6 million fractures are sustained in EU each year with a 5–20% resulting in delayed or impaired healing [4]. Delayed healing is often associated with chronic symptoms such as pain, functional and psychosocial disability. Developing novel therapies to enhance bone formation, to shorten the healing time, prevent non-unions and improve the quality of life is an urgent medical need [5–7].

Bone Morphogenetic proteins (BMPs) are members of the TGF-β superfamily of growth and differentiation factors that exert their function by binding to surface specific BMP receptors of mesenchymal stem cells (MSC) and cause them to differentiate to cartilage and bone-forming cells. Attempts to biologically support fracture healing were based on the use of either recombinant human bone morphogenetic protein 2 (rhBMP2) or recombinant human bone morphogenetic protein 7 (rhBMP7) [8]. These signalling molecules initiate and accelerate bone formation. However, their marketed formulations include the use of bovine collagen carrier, which is immunogenic and inflammatory in clinical settings, and when used with large amounts of recombinant BMPs, causes side effects. In addition, although rhBMP2 and rhBMP7 may be efficacious in long bone fractures, such as the broken lower leg, they do not promote bone formation inside cancellous bone, such as hip or wrist fractures [9,10].

Only a few adjunctive therapies for acceleration of the fracture healing are currently being used in the clinical setting, including low intensity ultrasound [11–13], local therapy with rhBMP2 [14], a treatment of tibial non-unions with rhBMP7 [8] and PTH. However, PTH was recently shown to be ineffective in shortening time to cortical bridging in a randomized, double-blind study of 102 postmenopausal women with distal radial fractures [15].

Since more than 80% of all fractures in humans involve the trabecular bone, rhBMP2 has been tested in preclinical studies in animal models of trabecular bone fractures with variable success [16]. The use of rhBMP7 in corrective osteotomies of radial bone due to the impaired healing with angular deformity in patients was also unsuccessful [17]. There is thus a significant medical need for development of a novel osteogenic therapy that will offer effective healing based on medically acceptable components which need to be cost-effective and affordable for treatment interventions in bone regeneration, in particular at cancellous bone surfaces comprising the majority of bone injuries.

We have recently developed an autologous bone graft substitute (ABGS), a combination product consisting of an autologous blood coagulum (ABC) that serves as a carrier for rhBMP6 [18]. It is a potent stimulator of bone regeneration that is being developed as a novel treatment to be applied in a single dose directly to the fracture site to accelerate bone healing [19]. In animal models, ABGS showed excellent tolerance and safety profile, elicited no immune response (no swelling, redness, oedema) and was locally retained (no systemic exposure). In the efficacy models, ABGS reproducibly induced new bone and restored bone defects in the dose range 50–100 μg rhBMP6/mL ABC [18].

In the present study, we present clinical results of this novel therapy for bone regeneration and its assessment following intraosseal administration in adult patients who sustained a distal radial fracture (DRF). The study was the First in Human (FiH) trial comparing a single dose of ABGS (rhBMP6/ABC) and placebo (PBO) to standard of care (SoC) in a randomized Phase I trial. The study aimed to assess primary safety and secondary efficacy of ABGS in patients with unilateral dorsally angulated closed fracture (Collies fracture) of the distal radius, requiring closed percutaneous stabilization by Kirschner wires.

2. Materials and methods

2.1. Investigational medicinal product (IMP)

The investigational therapy in the trial was rhBMP6 within ABGS which was compared to placebo (PBO) and standard of care (SoC) as reference therapies. Genera Research Ltd., a biotech company, provided rhBMP6 and PBO, ensured their characterisation and activity testing according to GMP principles, including the drug substance and formulated drug product containing rhBMP6 as an active pharmaceutical ingredient. Competent authorities evaluated regulatory compliant documentation and issued their approvals for use of the product in human clinical trials. The activity of rhBMP6 was verified by the in vitro testing - mouse C2C12-BRE-Luc BMP reporter cell assay, as previously described [18,20,21].

2.2. Intervention

Allocated local treatment was delivered by injection to the fracture site as a single dose of 250 μg/mL ABC after fracture reduction and percutaneous fixation with Kirschner wires and external fixators (if applicable). After the procedure the volar plast was added in duration of 5 weeks and after removal of the immobilization physical therapy started which is standard hospital procedure. In addition to intervention, all patients received the standard of care (SoC).

The ABGS and PBO implants were prepared using patient's own blood collected from the cubital vein as described in Fig. 1A–D. ABGS was prepared with 250 μg of rhBMP6 using 1 mL of freshly collected blood in a sterile cabinet located in the operating room (OR) as previously described [22]. PBO was prepared using 1 mL blood where only excipients in the same amount as in rhBMP6 formulation were added ensuring blinding. The quality control of each implant demonstrated uniformity of the final investigational product with physical appearance and rheological properties of ABGS (rhBMP6/ABC) and placebo (PBO) implants being the same. The implants were left to coagulate in syringes and were characterized prior to use as: red to deep red colored, cylindrically shaped, coagulated mass that detaches from the syringe wall after pulling the syringe clip. The administration was performed 60–90 min after the start of preparation.

The syringe with investigational therapy was given to the orthopaedic surgeon by the pharmacist into the sterile field. The ABGS and PBO were delivered by transcutaneous injection and administered under fluoroscopic control through a 14G needle into the fracture gap by the dorsal approach (proximal-to-distal direction) (Fig. 1E and F).

2.3. Clinical study design

The study was designed as a two-stage, one dose-level, placebo (PBO) and standard of care (SoC) controlled randomized trial to evaluate the safety and efficacy of ABGS and PBO administered in distal radial fracture site of patients who were elected to undergo a DRF treatment procedure. Trial duration was 26 weeks. The study was conducted at two study sites (Clinical Hospital Center Sisters of Mercy in Zagreb and University Clinical Center Sarajevo) under trial registration number: EudraCT 2014-005101-21. The total number of patients enrolled was 32 with a final assignment SoC: PBO: ABGS 5: 7:7 in Phase IA study, and 6:4:3 in Phase IB study. Phase IA was intended to obtain “First-in-Human” safety data on a total of 19 patients, 7 of whom were randomized to ABGS, 7 to PBO and 5 to SoC. As this was the first BMP-based product tested on the trabecular bone surface for this indication, patient enrolment was gradual. Enrolment was designed in

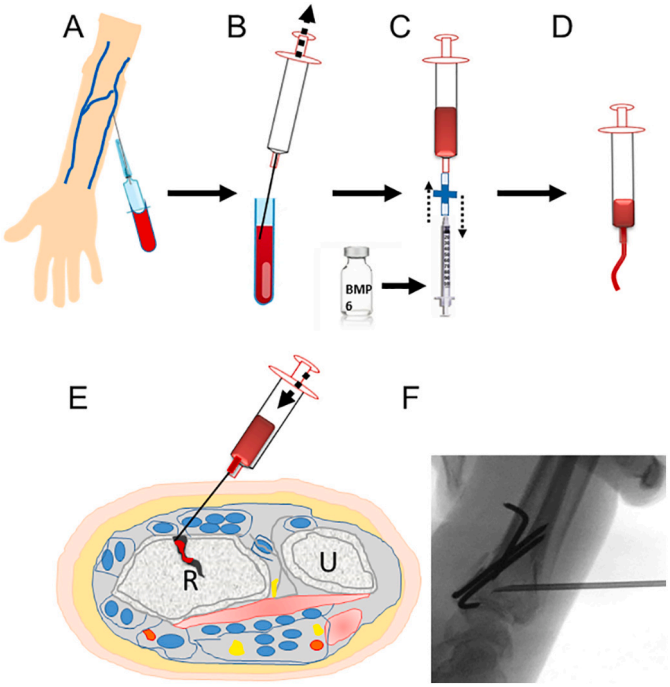

Fig. 1. IMP (PBO and ABGS) used in this study: preparation, appearance and application. A. Blood withdrawal from the patient's cubital vein. B. Aspiration of the blood from the vacutainer. C. Mixing the components of the IMP. D. Incubating the syringe with IMP at room temperature, quality control of the final product: red colored, cylindrically shaped coagulum that detaches from the syringe wall, coagulum is homogenous, malleable, cohesive and ready for injectable implantation. E. IMP injected dorsally into the distal radial fracture site. F. Fluoroscopic intraoperative observation of transcutaneous injection of IMP into the fracture gap by the dorsal approach. Steps B–D are performed in the sterile cabinet by the trained pharmacist while the surgeon performs the application to the patient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clusters, 3 clusters with 3 patient each (cluster 1, 2 and 3) and 2 clusters (cluster 4 and 5) with 5 patients each. If (any) patient was randomized to SoC, the next one was enrolled without a delay, but when a patient was assigned to ABGS or PBO, the next patient was enrolled only after at least 48 h if no safety issues have elapsed. The time interval between the clusters was 7 days that included clinical (local and systemic) evaluation and safety laboratory assessments at days 1, 2, 3 and 7. Phase IB proceeded upon getting an approval from Independent Data and Safety Monitoring Board (IDSMB) which was responsible for the overall assessment of safety based on clinical, laboratory and X-ray data from the first 19 patients. A total of 13 patients were enrolled to Phase IB, 3 of whom were randomized to ABGS, 4 to PBO and 6 to SoC. Following last patient last visit all data collected was presented to IDSMB for review.

2.4. Sample size determination

Since this trial evaluated ABGS in a FiH setting and was focused on safety, estimation of systemic exposure and exploration of efficacy, no single criterion was used to determine the sample size. Rather, sample size was determined in order to accommodate for several objectives.

The 32 patients (with 6 repeated early assessments) provide > 80% power to detect mentioned differences in the local status scores or laboratory test results between any two groups at two-sided alpha = 0.1, even if variability is high. Occurrence of a clinically relevant adverse effect in 1/10 ABGS-treated patients would strongly indicate it as a common safety issue – probability of seeing 1 such event in 10 patients falls to < 50% with true incidence ≤5%. This applies to any serious adverse event that would occur only in the ABGS (or PBO) group and not in the SoC group.

The exploratory evaluation of efficacy was based on proportion of "successes".

2.5. Inclusion and exclusion criteria

Patients for the DRF study were included if they had a current diagnosis of unilateral dorsally angulated closed fracture of the distal radius within the past 72 h needing closed reduction and stabilization with Kirschner wires, without open surgery. Both males and females at age ≥18 years were included, willing and able to be confined to the hospital/inpatient unit for at least 48 h postoperatively, and to comply with all other follow-up procedures. Females of childbearing potential had to be negative for the urine pregnancy test prior to the randomization. Patients were excluded from the study if they had any of the following: a previous fracture or bone surgery in the currently fractured distal forearm; joint diseases that affect the function of the wrist and/or hand of the injured arm; previous treatment with rhBMPs; clinically significant hepatic disease or other abnormalities in screening laboratory tests; an uncontrolled medical condition, including bone metabolic, renal, endocrine, hepatic, respiratory, cardiovascular, hematologic, immunologic or cerebrovascular disease, and malignancy; history of symptomatic nephro- or urolithiasis within two years; history of diabetes mellitus; treatment with an investigational drug within 6 months preceding the first dose of the study medication; screening ECG demonstrating at least one of the following: heart rate > 100 bpm, QRS > 120 ms, QTc > 430 ms (males), QTc > 450 (females) or PR interval > 220 ms, use of corticosteroids within 7 days prior to surgery and postoperative for the duration of the study; evidence of human immunodeficiency virus (HIV) antibody; hepatitis B infection within the past year or history of non-adequately treated hepatitis C infection; drug or alcohol abuse; donation of blood in excess of 500 mL within 56 days prior to and 1 month following surgery; and current participation in any other clinical trial. The patients provided written informed consent prior to any study procedure and the trial was approved by the competent authorities in Croatia and Bosnia and Herzegovina.

2.6. Safety outcome measures

Safety was assessed continuously throughout the trial based on clinical signs, serial vital signs assessments, laboratory assessments and spontaneously reported adverse events. Local safety/tolerability was specifically assessed by clinical inspection (e.g., signs of inflammation), pain and functional assessment, as well as radiological assessment with a particular focus on possible soft tissue ossification. Additionally, the potential for anti-rhBMP6 antibodies formation was investigated in PBO and ABGS patients at weeks 13 and 26.

MedDRA versions 19.0, 19.1, 20.0, 20.1, 21.0, 21.1, 22.0, 22.1 and 23.0 were used for the coding of adverse events in the cumulative study period. Any adverse events and serious adverse events occurring at any given time during the trial (clinical, laboratory, radiological), local or systemic were regularly evaluated by the independent monitor, pharmacovigilance provider and IDSMB. All enrolled patients were followed-up for 13 weeks with post-follow-up visit at week 26.

2.7. Collection of plasma and serum samples

For pharmacokinetic (PK) analyses venous blood (2 × 3 mL) was withdrawn into the EDTA-coated vacutainer following the standard procedure. Blood was collected according to the following schedule: time point 0 (prior to the application of rhBMP6), 15, 30, 45, 60, 90 min, 2, 4, 6, 12 and 24 h after application. For patients receiving the standard of care (SoC), blood was collected only at time point 0. Plasma was obtained by centrifugation and aliquoted in two cryo vials,

containing 800–1000 μL of plasma each. Samples were stored at −80 °C until analysis.

For antibody analysis, 5 mL of venous blood was withdrawn from the patient of concern into the plain vacutainer following the standard procedure. Blood was collected at time point 0 (prior to the application of rhBMP6), 13 and 26 weeks post-dose. For patients receiving the standard of care (SoC), blood was collected only at time point 0. Serum was obtained by centrifugation, aliquoted in two cryo vials and stored at −80 °C until analysis.

2.8. PK analysis

The presence of rhBMP6 in plasma of human patients was assayed by the ELISA method using the modified procedure of the human BMP6 DuoSet ELISA Development kit (DY507, R&D Systems, USA) using range of rhBMP6 standard concentrations 0.9 ng/mL – 1 μg/mL using as internal reference standard material batch F15227 produced by Genera Research. Absorbance readings were made at 450 nm using Perkin Elmer Multimode Plate Reader Victor™ X3 microtiter plate reader. For dilution of reference standards and test samples, plasma pool from several different human samples was used as a diluent. The in-house validation of the ELISA method was performed by validating the following parameters: standard calibration curve, matrix selection, specificity, selectivity, accuracy and precision, dilution linearity and stability [23].

2.9. Anti-rhBMP6 antibody analyses

For detection of anti-rhBMP6 antibodies an indirect ELISA method was used. Microtiter plates were coated overnight at +4 °C with 100 ng/mL rhBMP6. Each serum sample was diluted in 6 different dilution points (minimal dilution point 1:100). Biotinylated goat anti-human IgG H&L (Abcam ab6857, UK) diluted 1:10000 was used as secondary antibody. Each plate included the negative serum pool (composed of 10 human naïve serum samples obtained form 10 different individuals) diluted at the same ratio as the clinical samples. During the in-house validation of the indirect ELISA, the following parameters were validated: antigen coating concentration, diluent selection, recovery, secondary antibody concentration, minimal sample dilution, assay cut point and assay variability. The assay cut point was calculated with the formula mean + 1.645 × SD, where 1.645 is the 95th percentile of the normal distribution [24].

2.10. Efficacy outcome measures

Bone healing acceleration was assessed using CT and radiographic analyses. Anteroposterior (AP) and latero-lateral (LL) radiographs of the injured bone were taken prior to surgery, within 24 h after surgery, and at weeks 5, 9, 13 and 26 following surgery. CT of the injured bone was performed prior to surgery and at weeks 5, 9, 13 and 26. Radiographs/CT scans taken before the surgery and at weeks 5, 9 and 13 were blindly evaluated by three independent evaluators (two orthopaedic surgeons and one radiologist).

Two phases of bone-healing acceleration were evaluated as per study protocol; the early and the late phase. The early phase was characterized by callus formation which was evaluated from the X-ray and CT scans at week 5. Based on radiographic images taken on weeks 9 and 13 accelerated late bone healing was evaluated. Acceleration of bone healing was also measured by two additional parameters post hoc; fracture line closure and trabecular resorption area at weeks 5, week 9 and week 13 following surgery. Each evaluator provided a single assessment based on CT and X-ray projections for four different radiology parameters (formation of callus, trabecular fracture line closure, rebridgement of cortices and trabecular resorption area) graded using the following score system: 2 representing treatment success, 1 partial success and 0 treatment failure. More precisely the scores were defined as following: formation of callus: 2 represented “clearly visible callus”, score 1 “some callus” and score 0 “no visible callus”; trabecular fracture line closure: 2 represented “significant (complete) closure”, 1 represented “some closure” and 0 score “no trabecular fracture line closure”; rebridgement of cortices: 2 represented “rebridgement in at least 3 out of four cortices”, 1 represented “rebridgement in two out of four cortices or in one out of four cortices with disappearance of the fracture line in at least two additional cortices” and 0 “less than partial success”; trabecular resorption area: 2 represented “no resorption”, 1 represented “some resorption” and 0 “significant resorption”. The total scores of 3 evaluators were summed up and statistically analysed in two ways. For the first analysis, the total radiology score for each evaluator presented the sum of scores of each individual radiology parameter at all time points (week 5, week 9 and week 13) and the final score for each patient represented the calculated mean of total scores of all 3 evaluators. For the second analysis, the total radiology score for each evaluator represented the sum of scores of all four parameters determined at each time point (week 5, week 9 and week 13). The final score for each patient represented the calculated mean of total scores of all 3 evaluators. Statistical evaluation was performed as described in Section 2.14.

2.11. Study oversight

Study sponsor's (Genera Research) partners and investigators oversaw the execution of the protocol and planned the analyses before unblinding of the treatment assignments. The sponsor held the data and performed the analyses, designed the protocol and was responsible for the management and the quality control. The important changes to the initial protocol included change of two exclusion criteria: history of diabetes mellitus instead of diabetes mellitus, and NSAIDs were excluded as prohibited medication.

The study sites were supervised by study monitor according to the Monitoring plan. This included accurate and complete recording of data on Case Report Forms (CRFs), source documents, drug accountability records, Investigator site file (ISF) completeness, adverse events documentation, and training sessions of study team in accordance with Good clinical practice (GCP) principles.

2.12. Randomization and blinding

The randomization was implemented during the product labelling (at Contract Research Organization) ensuring the random allocation of designated treatment as per trial protocol by means of a simple randomization method. The vials containing lyophilized rhBMP6 and the PBO excipients could not be distinguished because both the vials and the contents had the same visual appearance and this was also the case with the final ABGS and PBO implants. The participants were enrolled into the trial by study investigators and allocated to study treatment by means of sequentially numbered, opaque and sealed envelopes (SNOSE) stored in a secure container. The trial was assessor blind (no-treatment control can be identified), but PBO and ABGS were double-blind (DB). The patients, investigators, site personnel and the pharmacist were blinded to the treatment assignment. The blinded study coordinator randomized participants following eligibility confirmation, and was responsible for securing the randomization envelopes in a secure location. Blinded pharmacist prepared all treatments based on randomization. Data analyses were performed by blinded evaluators.

2.13. Data management

In this study, the paper CRF was used to collect the subject data. Data recorded on CRFs were verified by checking the CRF entries against source documents in order to ensure data completeness and accuracy. The principal investigator ensured that CRFs and source documents of subjects enrolled in the study were available to monitor at

each monitoring visit, and also that all potential questions were answered and eventual inaccuracies corrected. Radiological outcomes were assessed by Radiology evaluation board consisting of an independent expert panel of two orthopaedic surgeons and one radiologist.

2.14. Statistical analysis

Kolmogorov-Smirnov test was used to assess data distribution and according to the results appropriate parametric and non-parametric test were used during the statistical analyses. Fisher-Freeman-Halton test was used to analyse differences in categorical variables like gender and operated arm between the three treatment groups (ABGS, PBO and SoC). Kruskal-Wallis test was used to analyse differences in age, height, weight, BMI and NRS scale between the groups. Friedman test was used to analyse pain dynamics from day 1 to week 13 for each group. One way ANOVA with post-hoc Bonferroni analyses was used to compare scores from different parameters between the groups. ANOVA for repeated measures was used to analyse differences in radiographic score dynamics between treatment groups at week 5, 9 and 13. All P values below 0.05 were considered significant. Statistical software IBM SPSS Statistics version 25.0 was used in all statistical procedures and for generating graphical representations of data. P value in Fig. 5 was drawn in Microsoft Powerpoint.

The effect sizes (Cohen's d) were calculated for pairs of trial arms for each radiographic parameter as well as for all three time points according to recommended methodology [25,26] using the online calculator (https://lbecker.uccs.edu/effect-size). The effect size d of 0.2–0.5 signified low effect size, 0.5–0.8 medium effect size, and $d > 0.8$ high effect size.

2.15. Study approval

The clinical study protocol was approved in Croatia by the Central Ethics Committee (No. 381-15/60-14-04) and by the Ministry of Health (No. 534-03-2-2/1-14/02). Clinical study protocol was approved in Bosnia and Herzegovina by the Ethics Committee (No. 0302-15831 and No.0302-15832) and by the Regulatory agency (No. 08-07.5-3723-1/16). All participants provided written informed consent prior to their inclusion in the study. The study was conceived, designed, initiated and performed by the academic investigators. The authors confirm the accuracy and completeness of the data and analysis and the fidelity of the study to the protocol. All the authors agreed on the final version of the manuscript prior to publication.

3. Results

The IMP used in this study (PBO and ABGS) was prepared and verified for quality by the study pharmacist and was applied to the DRF gap by the surgeon as presented in Fig. 1.

3.1. Demography of study participants

The trial was conducted at two study sites (Clinical Hospital Center Sisters of Mercy, Zagreb, Croatia and University Clinical Center Sarajevo, Bosnia and Herzegovina) from December 2015 until January 2020. The study enrolled 32 patients; 28 at Zagreb site and 4 at Sarajevo site. 32 participants received assigned treatment as per original study plan. The trial terminated prematurely due to the slow enrolment, and Phase IB and Phase II were not completed. Of 32 enrolled patients, 2 patients were lost to follow-up, and 2 patients withdrew their consent (Fig. 2).

The mean age of the patients in SoC was 54.5, in ABGS 53.5 and in PBO 61.0. There were no major differences in location of the fracture with respect to left or right limb between groups (SoC, ABGS and PBO) (Table 1). The trial enrolled significantly higher number of female participants (28 females vs. 4 men), but there were no significant differences in gender between the groups ($P = 0.512$).

3.2. Safety and adverse events

During the treatment and trial duration, no adverse reactions were reported. On the other hand, for 27 of 32 included patients, adverse events were reported (either serious (SAE) or non-serious (AE)). There was a total of 3 SAEs reported and all of them were reported at Zagreb site. In the SoC group 2 SAEs (urinary tract infection and myocardial infarction) were reported and in ABGS group 1 SAE (upper limb fracture) was reported. None of these SAEs were related to the investigational drug as judged by the investigator and the IDSMB.

All of the AEs (82 events in 27 patients) were rated mild to moderate and assessed as not related to the treatment by the investigator. In SoC group the following AEs were recorded: anaemia (1×), arthralgia (1×), bradycardia (2×), headache (1×), hypertension (10×), hyperhidrosis (1×), pain (5×), pyrexia (2×), tachycardia (2×) and vomiting (1×), in ABGS group the following AEs were recorded: anaemia (2×), dizziness (1×), headache (2×), hypertension (6×), pyrexia (2×), tachycardia (6×) and urinary tract infection (5×), while in PBO group the following AE were recorded: bradycardia (2×), headache (1×), hypertension (15×), hypotension (1×), hyperglycaemia (1×), pyrexia (2×), tachycardia (9×) and vaginal infection (1×). No local site infection was observed in any of the patients included in the study.

3.3. Pain measurement in DRF study patients

The pain in SoC patients, ABGS and PBO treated patients was evaluated by a numeric rating scale (NRS) (Fig. 3). Only few patients had severe pain, while, on average, none of the mean values for the PBO and ABGS treated patients from day 1 until week 13 were above the moderate pain rating from 4 to 6 [27]. However, in SoC group the mean values at screening visit and 12 h following surgery were between 6 and 7, while at the other study visits none of the mean values were above the moderate pain. There was no significant difference between groups in any time point except at 12 h following surgery. Using the Kruskal-Wallis (with post hoc Mann-Whitney test) test we showed that ABGS patients at 12 h post-dose had significant lower pain rating compared to SoC group ($P = 0.007$). Using the Friedman test we showed that there was a difference in the pain dynamics during the period of 13 weeks following surgery in PBO ($P = 0.008$) and ABGS ($P = 0.034$) patients having lower levels of pain, while in SoC patients there was no difference in the pain dynamics ($P = 0.132$).

3.4. Pharmacokinetics of rhBMP6 and anti-rhBMP6 antibodies

During the ELISA validation, the low detection limit of rhBMP6 in plasma was determined to be 5 ng/mL. No plasma samples taken from patients participating in this study showed measurable amounts of rhBMP6 at any time point, meaning that the rhBMP6 concentration in plasma was lower than 5 ng/mL in all samples.

Serum samples taken from all patients showed no measurable amounts of anti-rhBMP6 antibodies at 13 and 26 weeks, respectively (data not shown).

3.5. Radiographs and CT evaluation of radial fracture healing

All patients were treated with Kirschner wires which were removed at week 5. In only one patient the external fixator was used as additional immobilization as per surgeon's judgment and removed at week 5 together with the Kirschner wires as per standard hospital procedure. The radiographic and CT images were evaluated by 3 independent readers and scores were given to formation of callus, trabecular fracture line closure, rebridgement of cortices and trabecular resorption area. Using one-way ANOVA with post-hoc Bonferroni test we showed that

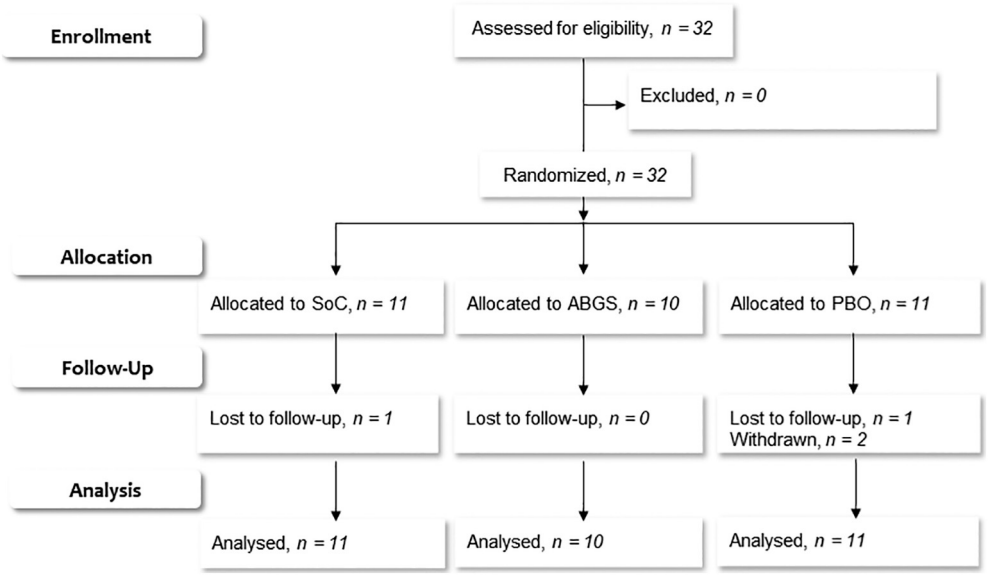

Fig. 2. CONSORT diagram of the clinical trial.

Table 1
Demographic characteristics of patients enrolled into the DRF study.

Characteristic	ABGS	PBO	SoC	P-value
Patients	10	11	11	–
Gender (male/female)[a]	0/10	2/9	2/9	0.512
Arm (left/right)[a]	6/4	5/6	4/7	0.611
Age in years[b]	53.5 (14.7)	61 (11.9)	54.5 (16.2)[c]	0.426
Height in cm[b]	166.7 (7)	170.2 (6.3)	168.5 (7.6)	0.491
Weight in kg[b]	76.1 (12.9)	73.4 (10.9)	71.6 (13.2)	0.610
Body Mass Index* as kg/m^2	27.3 (3.9)	25.2 (4.8)	25.3 (3.11)	0.290

[a]Fisher-Freeman-Halton's test, [b]Kruskal Wallis test, [c]values are means with SD in parentheses.

there was a significant difference in rebridgement of cortices and in the total score of all 4 parameters in ABGS patients compared to PBO, respectively (P = 0.005, P = 0.038) while there was not a significant difference to SoC treated patients, respectively (P = 0.069, P = 0.395). In ABGS patients the mean of total scores from all evaluators were significantly higher as compared to scores of patients treated with PBO (Fig. 4), while there was no significant differences as compared to SoC treated patients. However, when radiology parameters were evaluated based on the magnitude of difference in mean values (Cohen's d) medium to large effect sizes were observed comparing ABGS to PBO and SoC group, respectively: trabecular fracture line closure (1.17, 0.69), rebridgement of cortices (1.45, 1.17), trabecular resorption area (0.88, 1.04) and total score (1.15, 0.87).

The ABGS therapy showed accelerated early healing at week 5 and late healing at week 9 (Fig. 5). ABGS had the highest total score at week 5 with mean (SD) of 3.87 (0.94), and at week 9 5.87 (0.63) compared to the PBO and SoC treated patients, while at week 13 in all three groups total score was around 6.5. PBO and SoC groups had the same total scores at all three time points. There was no significant differences between the groups at all three time points. However, analysis of total scores at week 5 and week 9 showed a significant difference in ABGS group as compared to SoC and PBO groups (P = 0.027). Additionally, the effect size (Cohen's d) of accelerated healing was pronounced at week 5 and 9 and not present at week 13, with ABGS group being superior to PBO and SoC groups, respectively: week 5 (0.94, 0.94) and week 9 (1.01, 0.94).

All measured parameters in all treated patients were similar at week 13, suggesting that healing outcomes reached equivalent results to all treatment groups. The closing of the fracture line was followed by X-rays and its progress is shown by arrows from day 0 to week 26 (Fig. 6). The fracture line healed at week 9 in patients treated with ABGS and at week 13 in PBO and SoC treated patients (Figs. 5 and 6). The cortical fracture line was closed at week 9 in ABGS treated patients (Figs. 5 and

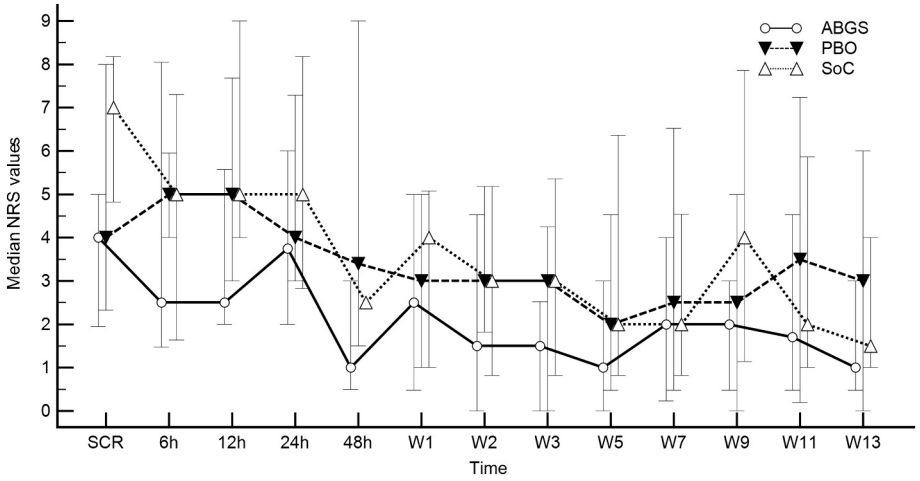

Fig. 3. Numeric Rating Scale (NRS) for pain in SoC (n = 11), ABGS (n = 10) and PBO (n = 11) treated patients following DRF surgery. P values for each measured time were calculated using Kruskal Wallis test, while difference in dynamics between groups was analysed with Friedman test. ABGS treated patients at 12 h post-dose had a significant lower pain rating compared to SoC (P = 0.007) treated patients. There was a difference in pain dynamics in PBO (P = 0.008) and ABGS (P = 0.034) patients during the period of 13 weeks following surgery. In SoC treated patients there was no difference in the pain dynamics (P = 0.132). SCR (screening), W1–W13 (Week 1–Week13).

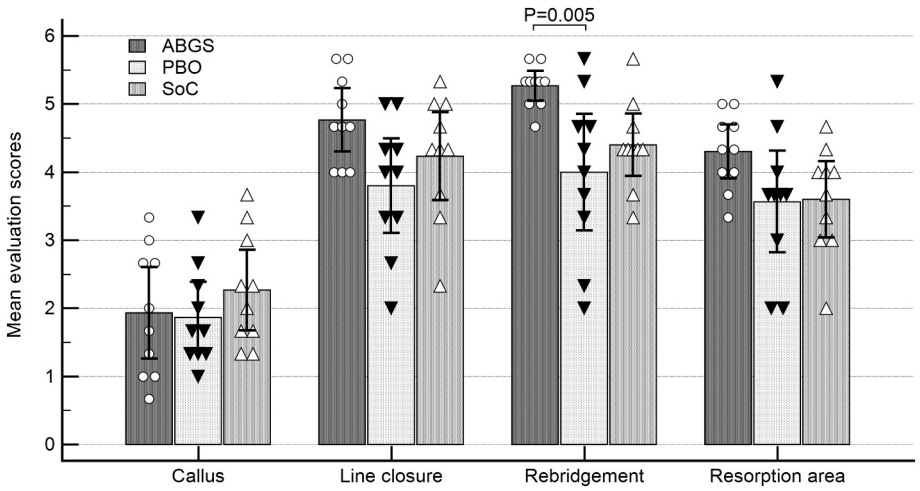

Fig. 4. The total radiology score for each evaluator represented the sum of scores of each parameter; Formation of callus (Callus), Trabecular fracture line closure (Line closure), Rebridgement of cortices (Rebridgement) and Trabecular resorption area (Resorption area) in all time points (weeks 5, 9, 13). The final score for each patient represented the calculated mean of total scores of 3 evaluators. The mean (SD) in ABGS treated patients was 16.27 (1.70), in PBO group 13.23 (3.33) and in SoC group was 14.5 (2.32). There was a significant difference in rebridgment of cortices in ABGS treated patients as compared to PBO (P = 0.005).
PBO n = 10, ABGS n = 10, SoC n = 10.

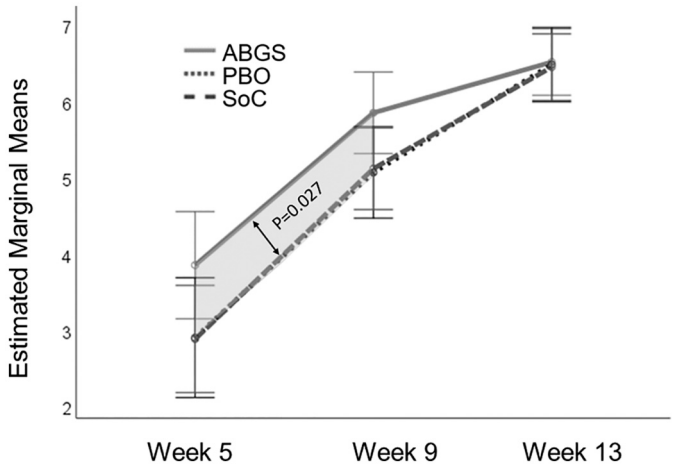

Fig. 5. The total radiology score for each evaluator represented the sum of score of all four evaluated parameters (formation of the callus, trabecular fracture line closure, rebridgement of cortices and trabecular resorption area) determined at weeks 5, 9 and 13. The final score for each patient represented the calculated mean of total scores of 3 evaluators. ABGS treated patients had the highest total score at week 5 (mean (SD) = 3.87 (0.94)), and week 9 (mean (SD) = 5.87(0.63)) compared to the PBO and SoC treated patients, while at week 13 in all three groups total score was around 6.5. PBO and SoC groups had the same total scores at all three time points. There was no statistical differences between the groups, however analysis of total scores at week 5 and week 9 showed a statistical difference in ABGS group as compared to SoC and PBO treated patients (P = 0.027).

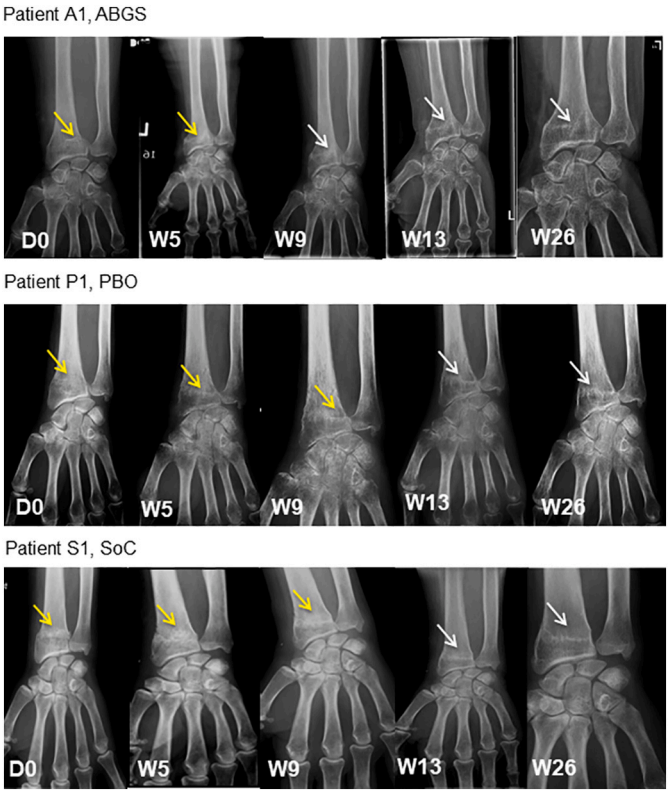

Fig. 6. X-ray images from representative DRF patients: treated with ABGS, PBO and SoC from day 0 to week 26. Yellow arrows represent the fracture line, and white arrows represent the fracture line closure indicating bone healing. In patient A1 fracture line healing was completed at week 9, while in patients P1and S1 the fracture line contour was fully remodeled and the bone integrated at week 13. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

7), and the trabecular bone defect filing of the radial metaphysis was more advanced in ABGS than in SoC and PBO treated patients (Figs. 4 and 7) which was also quantified as described in Fig. 4.

4. Discussion

We present here the results of a First in Human clinical testing of the safety, tolerability and efficacy of ABGS in patients with distal radial fracture. This was a randomized, controlled and quadruple blinded trial from the perspective of patients, pharmacists, surgeons and evaluators except for the SoC trial arm. We have shown that a novel autologous bone graft substitute (ABGS) containing rhBMP6 applied within autologous blood is safe and has good tolerability on the trabecular bone surface following application to closed distal radial fractures. We show that ABGS, when administered as injectable coagulum to the fracture site, does not release rhBMP6 in systemic circulation and does not generate anti-rhBMP6 antibodies. Additionally, ABGS induced new bone formation with an accelerated rate as compared to placebo in the fracture gap of patients who underwent a distal radial fracture (DRF) treatment procedure.

Demonstrating the safety is of utmost importance for the clinical application of any novel therapy. The safety profile of ABGS in this trial was shown to be superior to its forerunners rhBMP7 and rhBMP2. RhBMP2 (InFuse) and rhBMP7 (OP1-Implants) osteogenic devices employ bovine-sourced collagenous matrix as a carrier. Bovine collagen causes inflammation and immune responses but also promotes the differentiation of mesenchymal stem cells into fibroblast phenotype

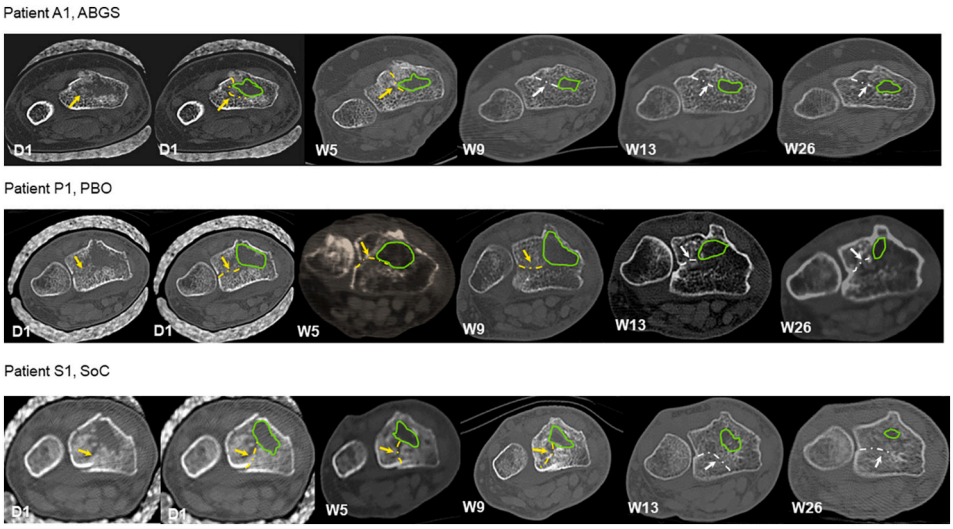

Fig. 7. CT images from representative DRF patients: treated with ABGS, PBO and SoC from day 1 (before the surgery) to week 26. Fracture line was indicated by yellow dashed line and the yellow arrow, while the closed fracture line was shown by white dashed line and the white arrow. Green circles from day 1 to week 26 represented the reduction of the trabecular resorption defect. Closure of the fracture line is seen at week 9 in patient A1, and at week 13 in patients P1 and S1. Reduction of trabecular resorption defect is more significant in A1 patient compared to SoC and PBO patients. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

more than to the osteoblasts. This requires the use of higher dose of BMPs which results in resorption of the trabecular bone. Furthermore, as rhBMP2 and rhBMP7 have a weak affinity to collagenous matrix, the protein distributes away from the implant and thus elicits unwanted safety issues, including the production of anti-BMP antibodies [28,29]. It is worth to mention that the application of rhBMP7 in bovine bone collagen in distal radial fracture resulted in adverse safety issues upon administration including local inflammation, redness, swelling and oedema in Phase I safety trial and therefore was discontinued [30].

ABGS, on the other hand, employs autologous blood coagulum fabricated from patient's own blood thus helping to reduce inflammation and immune response associated with animal derived collagenous carrier. The safety profile, as assessed here, shows that AGBS upon administration at the distal radial fracture site did not elicit any unwanted safety issues including no adverse reactions or swelling, redness of the skin, oedema or any distant soft tissue ossification or increased pain. In addition, no systemic rhBMP6 has been found following administration of ABGS, no antibodies against rhBMP-6 have been detected after 13 and 26 weeks and no observable systemic side effects were observed over the 26 weeks follow-up. We suggest that the tight binding of rhBMP6 to blood proteins enables its retention at the local injection site, slowing its release into the environment and uptake to target cells, thus eliciting the local bone stimulation effect. At the same time, this mechanism disables the biodistribution of rhBMP6 into the systemic circulation and prevents potential unwanted systemic effects.

Currently available therapies do not accelerate and enhance healing of acute long bone fractures, neither do they decrease the incidence of secondary interventions below 5–20% [2]. Based on the preliminary efficacy analysis, ABGS was able to accelerate bone healing during distal radial fracture early repair phase. This observation is in accordance with our recent findings that ABGS is capable of enhancing healing in the trabecular bone surface in Phase I/II clinical study in patients who underwent high tibial osteotomy [31].

Targeted delivery of an appropriately formulated (injectable) osteoinductive factor into the site of bone injury has not yet been achieved. By overcoming this bottleneck, many advantages in clinics could occur, such as in the percutaneous treatment of delayed or/and non-unions, minimally invasive spinal fusion, and accelerated healing of closed fractures. Recently recorded side effects in patients treated off-label with Infuse resulted in an alert issued by FDA to indicate that the usually applied rhBMP2 bone device can promote exuberant ectopic ossification and cause increased nerve compression, breathing and swallowing problems when used for cervical spine fusion [32]. The xenogeneic bovine collagen currently used for bone healing induces a strong inflammatory reaction with subsequent swelling and local skin redness, which does not allow the use of the devices in certain clinical settings such as periodontal surgery or cervical spine fusion procedures [33,34]. Previously, rhBMP7 (OP-1; Ossigraft) was used in comparison to the autogenous bone graft from the iliac crest in 30 patients with metaphyseal defects in the distal radius following corrective osteotomies after distal radial fractures [17]. The rhBMP7 on bovine collagen reduced the capacity for repair and resulted in osteolysis and healing at a slower rate than autogenous bone graft [17]. The negative effect of rhBMP7 bone device was eventually a result of a high rhBMP7 dose promoting resorption of the trabecular bone, bovine collagen induced inflammation and impaired differentiation of mesenchymal stem cells into new osteoblasts and bone trabeculi. Preclinical data with rhBMP6 using various rat, rabbit and sheep bone defect models demonstrated an accelerated healing, no inflammation response and efficacy of a low dose of rhBMP6 needed for successful outcome [18,35,36]. The same result is now translated to human use.

5. Conclusions

ABGS (rhBMP6/ABC) was found to be safe and well tolerated in patients with distal radial fracture. There were no observable systemic side effects over the 26-weeks follow-up. Plasma samples did not show measurable amounts of rhBMP6 at any time point during the 24 h sampling demonstrating local retention and no distribution into the systemic circulation. Additionally, no measurable amounts of anti-rhBMP6 antibodies were detected in serum samples taken from all patients participating in this study. ABGS is the first BMP-based product that uses autologous carrier for the delivery of bone promoting factor directly into the fracture site. ABGS is able to accelerate the bone healing during the early repair phase of distal radial fractures. We believe that a targeted delivery of a BMP with a physiological native carrier into the site of bone injury provides permissive environment for accelerated bone repair as well as protection against exuberant ectopic ossification causing unwanted side effects. In perspective, and based on some of our preclinical studies [18,22,35,36] a percutaneous treatment with ABGS may also help to repair the delayed and non-unions of diaphyseal fractures, minimally invasive spinal fusion, and accelerated healing of various closed fractures.

CRediT authorship contribution statement

Dragan Durdevic:Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization.**Tomislav Vlahovic**:Conceptualization, Methodology, Validation, Investigation, Data curation, Writing -

original draft, Writing - review & editing, Visualization.**Sanja Pehar:**Methodology, Writing - review & editing, Project administration.**Dina Miklic:**Investigation, Writing - review & editing. **Hermann Oppermann:**Conceptualization, Validation, Resources, Writing - review & editing, Supervision.**Tatjana Bordukalo-Niksic:**Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing.**Ismet Gavrankapetanovic:** Investigation, Writing - review & editing.**Mehmed Jamakosmanovic:** Investigation, Writing - review & editing.**Milan Milosevic:**Formal analysis, Writing - review & editing, Visualization.**Snjezana Martinovic:**Investigation, Writing - review & editing.**T. Kuber Sampath:**Conceptualization, Writing - review & editing, Supervision. **Mihaela Peric:**Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - review & editing, Visualization.**Lovorka Grgurevic:**Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - review & editing, Visualization.**Slobodan Vukicevic:**Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Visualization, Project administration, Funding acquisition.

Declaration of competing interest

LG, HO and SV have an issued patent US8197840 and licensed to Genera Research (GR). HO is an employee of Genera Research. TKS received grants and other from perForm Biologics during the study. MP, SM, LG, TBN, SV and HO collaborate on the development of a new drug for bone repair under the consortium of partners funded by EU HORIZON2020 (GA No 779340 [OSTEOproSPINE]). Other authors declare no conflict of interest.

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