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Serum albumin coating of demineralized bone matrix results in stronger new bone formation

Dénes B. Horváthy,[1,2] Gabriella Vácz,[1] Tamás Szabó,[3] Imola C. Szigyártó,[4] Ildikó Toró,[1] Boglárka Vámos,[1] István Hornyák,[1] Károly Renner,[5] Tamás Klára,[2] Bence T. Szabó,[6] Csaba Dobó-Nagy,[6] Attila Doros,[7] Zsombor Lacza[1,2]

[1]Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary
[2]Department of Orthopedics, Semmelweis University, Budapest, Hungary
[3]Department of Interfaces and Surface Modification, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary
[4]Department of Biological Nanochemistry, Institute of Molecular Pharmacology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary
[5]Department of Physical Chemistry and Material Science, Budapest University of Technology and Economics, Budapest, Hungary
[6]Department of Oral Diagnostics, Semmelweis University, Budapest, Hungary
[7]Department of Transplantation and Surgery, Semmelweis University, Budapest, Hungary

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Abstract: Blood serum fractions are hotly debated adjuvants in bone replacement therapies. In the present experiment, we coated demineralized bone matrices (DBM) with serum albumin and investigated stem cell attachment *in vitro* and bone formation in a rat calvaria defect model. In the *in vitro* experiments, we observed that significantly more cells adhere to the serum albumin coated DBMs at every time point. *In vivo* bone formation with albumin coated and uncoated DBM was monitored biweekly by computed tomography until 11 weeks postoperatively while empty defects served as controls. By the seventh week, the bone defect in the albumin group was almost completely closed (remaining defect $3.0 \pm 2.3\%$), while uncoated DBM and unfilled control groups still had significant defects (uncoated: $40.2 \pm 9.1\%$, control: $52.4 \pm 8.9\%$). Higher density values were also observed in the albumin coated DBM group. In addition, the serum albumin enhanced group showed significantly higher volume of newly formed bone in the microCT analysis and produced significantly higher breaking force and stiffness compared to the uncoated grafts (peak breaking force: uncoated: 15.7 ± 4 N, albumin 46.1 ± 11 N). In conclusion, this investigation shows that implanting serum albumin coated DBM significantly reduces healing period in nonhealing defects and results in mechanically stronger bone. These results also support the idea that serum albumin coating provides a convenient milieu for stem cell function, and a much improved bone grafting success can be achieved without the use of exogenous stem cells. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2015.

Key Words: serum albumin, bone, DBM, calvaria defect, stem cells

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INTRODUCTION

Allogeneic bone substitutes are extensively used in bone replacement interventions, such as, fresh-frozen, freeze-dried or demineralized bone. Although, allografts are generally safe, every step of the preparation process was shown to compromise bone-forming capacity.[1] For example, the harsh physico-chemical methods needed to remove antigens and reduce contamination, which also results in the reduction in osteogenic response of demineralized bone matrix (DBM).[2] One way of tackling this issue is to replace the missing ossification factors in the bone grafts before implantation. Recombinant bone morphogenic proteins (BMPs) can be loaded onto the surface of various bone substitutes and faster ossification can be observed in animal studies.[2–4] Although there is no question about the strong bone-inducing ability of BMPs, recent data gained from clinical use pointed out safety issues of these proteins.[5,6] Some of these issues may be related to the fact that BMPs are typically applied as single proteins, much like a classical drug; while optimal proliferation in the tissues normally requires several factors with varying concentrations. A balanced mixture of such growth factors is present in blood activated by an injury. Based on this idea, serum fractions, such as platelet rich plasma, are also

Correspondence to: D. B. Horváthy; e-mail: denes.horvathy@tissueengineering.hu
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under investigation in bone regeneration with mixed results so far.[7] Again, there is no question about the presence of osteogeneic factors in serum products, but it must be noted that these endogenous mixtures can also contain proinflammatory components which may interfere with the regeneration process. Therefore, it would be necessary to find an optimal serum fraction for a given type of bone defect, which can eventually lead to consistently better therapeutic outcomes. Serum albumin is a well-known proliferation factor for stem cells in culture and we hypothesized that this feature may also be beneficial as a bone graft additive.[8] In previous *in vitro* experiments we showed that freeze-dried serum albumin coating on human allografts provides a convenient milieu for mesenchymal stem cell (MSC) proliferation.[9] Albumin coated human allografts were also implanted in a rat nonunion femur model *in vivo*, where we found significant defect consolidation at four weeks after implantation.[10] At this time point, albumin coated allografts successfully bridged nonunion bone defects, while uncoated grafts failed. Later, we investigated the safety and surgical applicability of albumin-coated allografts in a human experiment, during which albumin-coated allografts were implanted in 10 cases of aseptic revision arthroplasty as a support for the metal prosthesis.[11] These experiments successfully showed the applicability of albumin coating and raised hope for better clinical outcome. However, it is yet unknown whether albumin coating alone results in a higher quality bone stock, which is the ultimate need for surgeons performing bone-replacement operations. In this study, we investigated stem cell attachment onto albumin coated demineralized bone *in vitro*. In addition, we investigated the *in vivo* bone forming capacity of the same material in a nonhealing calvaria defect in rats, followed by microCT and functional testing *ex vivo* to determine the strength and composition of the grafted tissue.

METHODS

Animals

Male Wistar rats weighing 250–300 g were used. The animals were maintained on lab chow and tap water ad libitum with 12 h day/night cycle in the animal facility of the Institute of Human Physiology and Clinical Experimental Research in Budapest. The investigation was approved by the local Animal Research Committee according to the guidelines for animal experimentation (Date of issue: 2009.10.07., Registration number: 22.1/2960/003/2009.)

Stem cell harvest and culture

Wistar rats were euthanized with urethane (5 mg/mL). Both tibiae and femora were cleaned of soft tissue and placed in 70% ethanol. Under a sterile hood, both ends of the bones were cut and the medulla was flushed out slowly with 10 mL culture medium (DMEM supplemented with 1 g/L glutamine, 1 g/L glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin) into 15-mL tubes. Bone marrow was centrifuged at 274*g* for 8 min at room temperature and was placed in 100 mm Petri dishes. After harvesting, BMSCs were maintained at 5% CO_2 level at 37°C. Two days later the culture was washed in Dulbecco's phosphate-buffered saline (PBS) and fresh culture medium was given every 48 h.

DBM preparation and albumin content

Male Wistar rats were euthanized with urethane (5 mg/mL). Parietal bone was identified, and cortical bone pieces were harvested with a 4 mm internal diameter trephine bur. The DBM was prepared by following the classical method originally described by Urist[12]: (1) defatting by methanol for 24 h. (2) Washing with PBS for 5 min. (3) Antigen removal by partial autolysis in 0.1 *M* PBS containing 10 mmol/L sodium azide and 10 mmol/L iodic acetic acid at 37°C for 48 h. (4) Decalcination in 0.6*N* hydrochloric acid at room temperature for 24 h. (Ca content in DBM was 0.1 ± 0.0 w/w%, untreated control 34.1 ± 0.2 w/w%). (5) Washing with PBS for 5 min. (6) Whitening in 3% hydrogen peroxide at room temperature for 36 h. (7) Washing in distilled water. (8) Freeze drying I: grafts were frozen at −80°C, freeze dried at −50°C overnight. (9) Freeze drying II: Freeze dried DBMs were soaked in 20% human albumin solution (Biotest, Hungary) and were frozen at −80°C, freeze dried at −50°C overnight.

To determine albumin content the Bradford protein assay was performed. Albumin coated DBMs were incubated in distilled water for one, two, and four days as separate groups. The solution was then incubated with 1 mL of Bradford reagent at room temperature. Calibration standard curve was made using 5–20 μL 0.5 mg/mL of human serum albumin (HSA) and 0.15*M* sodium-chloride for a final volume of 100 μL. The protein concentrations of the unknown samples were determined using a spectrophotometer and comparing the absorbance at the characteristic wavelength of 595 nm values against the standard curve. All chemicals were purchased from Sigma Aldrich, Budapest, Hungary.

In vitro attachment

Prepared DBM grafts were put into 96 well ultra-low attachment wells (Costar, Corning); 200 μL cell suspension containing 50.000 rat MSCs and stem cell media were cocultured with the grafts for 6, 12, and 24 h. Cellular attachment was measured with methyl-thiazole-tetrazolium assay (MTT, Sigma Aldrich, Budapest, Hungary).[13] Briefly, after 6, 12, and 24 h of coculture, media was removed and fresh media (200 μL) containing 5 mg/mL MTT (1:9) was added (37°C, 1 h). After 1 h of incubation, MTT containing media was removed and 200 μL propanol was added for 1 h under gentle shaking. The absorbance of MTT was using a spectrophotometer at the characteristic wavelength of 570 nm. The background was measured at 690 nm and the, absorbance values were correlated with the cell number.

Experimental protocol *in vivo*

In the *in vivo* experiment, nonunion bone defects were created on rat parietal bones. Bone formation was monitored

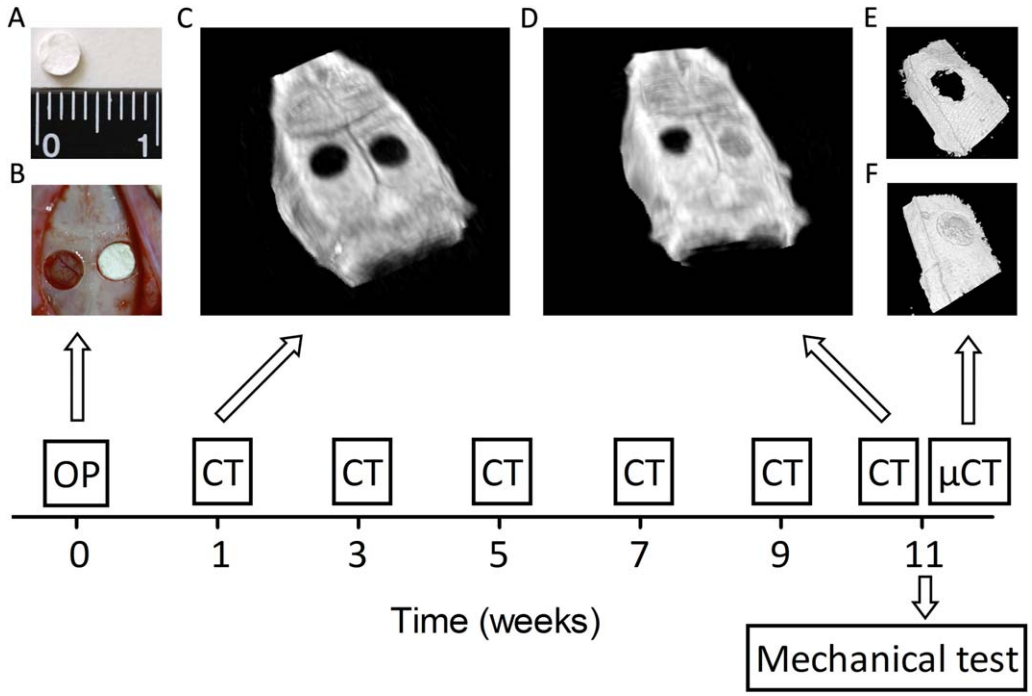

FIGURE 1. Experimental protocol. Panel A shows DBM before implantation (scale represents 1 cm). Panel B shows the surgical site and implanted graft on the right side. Panel C shows postoperative CT after 1 week with one defect in the center of the parietal bones (transparent graft on the right side). Panel D shows postoperative CT after 11 weeks with complete union on the right parietal bone. Panels E and F shows micro CT scans *ex vivo* after 11 weeks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

biweekly by computed tomography (CT) until 11 weeks postoperatively. On the 11th week, parietal bones were harvested for microCT scans and mechanical testing (Figure 1.). Three experimental groups were established. We tested albumin coated DBMs and compared them to uncoated DBMs or unfilled control (empty) defects. Eight animals were used each with two randomly filled parietal defects; six defects were filled with albumin coated allografts, six defects were filled with uncoated allografts, and four defects were left blank.

Surgical procedure

The surgical procedure was performed as described by Spicer et al. in Nature Protocols.[14] Under ketamine-xylazine (100–10 mg/kg, Richter Gedeon, Budapest, Hungary - Sigma Aldrich, Budapest, Hungary) anesthesia, and after proper disinfection, the skin was incised over the parietal bone of male Wistar rats. Periosteum was carefully elevated from the bone. Bone defects were created using a 4 mm trephine bur (external diameter) on both parietal bones. DBM were used to fill the defects and the periosteum was united in the midline with a thin absorbable suture (6-0 Vicryl, Johnson & Johnson, Janssen-Cilag, Hungary).

In vivo bone formation

Computed tomography. Under ketamine-xylazine anesthesia, CT was performed after 1, 3, 5, 7, 9, and 11 weeks postoperatively with a Phillips Brilliance 16 Slice CT machine (Philips International B.V. Amsterdam, Netherlands). Axial slices were obtained with 120 kV and 300 mA (slice thickness: 0.8 mm, increment 0.4 mm, collimation 16 × 0.75 mm, rotation time 0.75 s, pitch: 0.4).

Remaining bone defect. Area of the remaining bone defect was measured from the CT scans [Figure 3(A)]. Windows were set to visualize rat bone without soft tissue background (2572/1595 Window/Center). The remaining bone defect was calculated on an enlarged reconstructed image, with free hand technique. The area of the bone defect (mm^2) was converted to percentage and values were compared with the original defect size (100%).

Densitometry. Images from the CT scan were analyzed to determine the density of the bone defect [Figure 3(B)]. Circular region of interest (ROI) was set on the first postoperative week for every defect. To follow the changes in density, the identical ROI was used on the healing bones in every analysis.

Ex vivo micro CT

The harvested parietal bones were scanned using a micro CT scanner (Skyscan 1172 X-Ray microtomograph, Kontich, Belgium) at 59 kV, 167 μA with a 23 μm^3 isovolumetric pixel size. Image analysis was performed with CTAn software in the following way: the center of the bone defect was determined on the horizontal and coronal images. On coronal images a circular ROI with 80 pixels of radius were drawn and 100 slices were selected cranial and caudal from the center. As described previously, bone volume per tissue

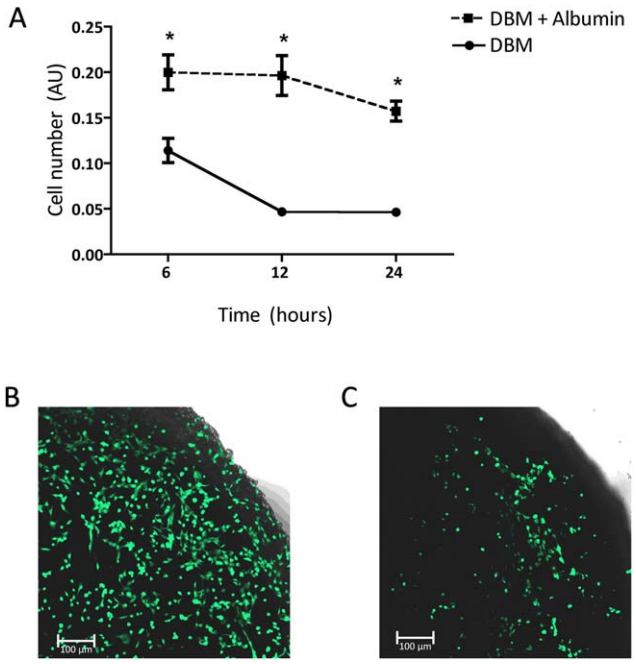

FIGURE 2. Cell attachment on the demineralized bone surface *in vitro*. Panel A shows absorbance values (arbitrary unit) after 6, 12, and 24 h after cell seeding. Albumin coated group presented significantly higher cell number after each time point (Two way ANOVA, Bonferroni posttests, $p < 0.001$). Panel B shows a representative image of albumin coated DBM after 12 h. Panel C represents an uncoated graft after 12 h. Green color (calcein) represents living cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

volume (BV/TV) was calculated to determine the relative bone content (%) in the defect area and in uninjured parts.[15]

Mechanical push-out testing

Mechanical push-out testing was performed with an Instron 5566 device (Instron, Norwood, MA) according to Kretlow's method.[16] A custom-made holder was created to hold the parietal bone. The tissue samples were gently fixed between two flat surfaces with a 5 mm hole in the middle. The graft was guided to the center of the holder. To determine the breaking force, the bone tissue was loaded with a 2.5 mm diameter rod with a flat bottom and rounded edges at 10 mm/min cross-head speed, whereas load-displacement data was registered in every 100 ms. The force was registered during pressing through the rod in the bone. Peak breaking force (N) of the samples were identified by the highest force measured. The slope of the triangular shaped load-displacement curves represented material stiffness (N/mm^2).

Statistical analysis

All of the values are reported as the means ± SEM. The statistical analysis was performed with t-test, One-way and Two-way ANOVA with Bonferroni multiple comparison tests using the GraphPad Prism statistical software. Probability values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant.

RESULTS

Albumin content

Albumin dissolution was measured after one, two and four days. After one day of incubation, the dissolved albumin content was 0.38 ± 0.05 mg/DBM. We found significantly higher albumin content when incubated for two days, but no significant change was observed after four days. (At two days: 0.70 ± 0.05 mg/DBM, at four days: 0.64 ± 0.05 mg/DBM). According to this the total amount of albumin was dissolved already at two days, determining the total serum albumin content of the DBMs.

In vitro attachment

Attached cell number was investigated after 6, 12, and 24 h. After 6 h of incubation, albumin coated grafts attached approximately twice as many cells as the uncoated grafts (Figure 2.). After 12 h of incubation, cell number significantly decreased on the surface of uncoated DBM. In contrast, the cell number did not change in the albumin coated group, even after 24 h (Figure 2.).

In vivo bone formation

Remaining bone defect. Bone formation was followed by biweekly measuring the area of the remaining bone defect. At the first week, the area of the remaining bone defect was measured [Figure 3(A)]. There was no significant difference in the experimental groups at this time point, representing close to 100% bone defect (empty: 9.7 ± 0.1 mm^2, uncoated: 11.2 ± 0.5 mm^2, albumin 10.6 ± 0.5 mm^2). After three weeks, the size of the bone defect decreased in every experimental group. The size of the remaining bone defect in the empty experimental group was $77.5 \pm 4\%$. The defects filled with uncoated grafts were $73.5 \pm 13.4\%$. In contrast, the size of the defects filled with albumin coated allografts was $43.8 \pm 14.0\%$, which was significantly lower than the sham experimental group [Figure 3(A)]. Albumin coated allografts showed even smaller bone defects at the fifth week. At this time point, uncoated and unfilled groups both showed more than 60% remaining bone defects. By the seventh week, the bone defect in the albumin group was almost completely closed ($3.0 \pm 2.3\%$), while the other two groups still had a significant bone defect (uncoated: $40.2 \pm 9.1\%$, empty: $52.4 \pm 8.9\%$). While the uncoated group showed a slow tendency to close the gap, the unfilled sham defects showed no significant reduction in the size of the remaining bone defect from the fifth week on.

Densitometry. Density of the bone defect was also measured [Figure 3(B)]. After one week a clear bone defect was seen in every experimental group. There was no significant difference between the experimental groups at this time point. As evidenced by the increasing density at 3 and 5 weeks, bone formation was observed in every experimental group. Albumin coated allografts showed higher density values compared to uncoated allografts and unfilled defects after the third week. By the seventh week, the density in the albumin coated group is significantly higher than in the

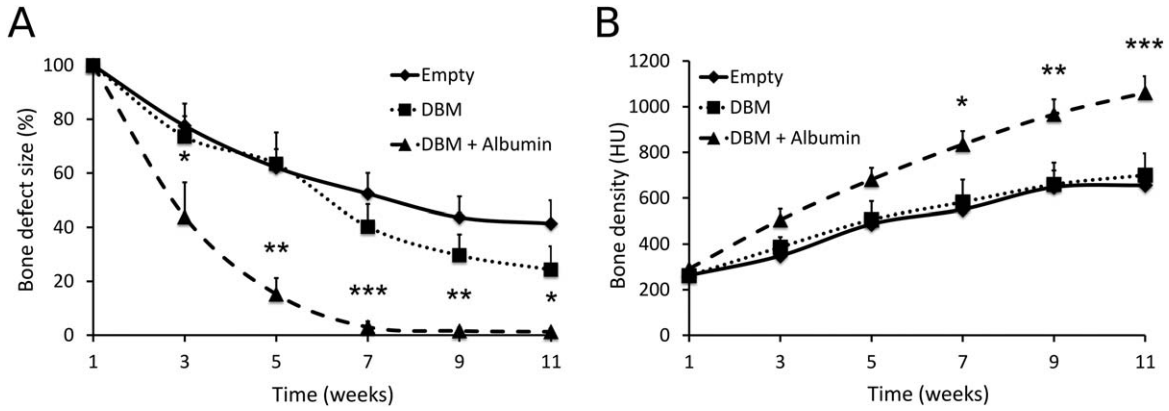

FIGURE 3. Measurements of new bone formation *in vivo*. Panel A shows the remaining bone defect in percentage relative to the bone defect after the first week. Albumin coated group show faster bone formation and full consolidation at week 7. Panel B shows density values of the defect area. Albumin coated DBM showed significantly higher density values from the seventh week compared with empty defects. (Two way ANOVA, Bonferroni posttests, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

other groups, the difference between them increased even further in time [at 11 weeks: empty: 655 ± 84 HU, uncoated: 700 ± 105 HU, albumin 1061 ± 79 HU, Figure 3(B)].

Ex vivo microCT

The BV/TV values of uninjured parietal bones were $36.1 \pm 1.7\%$, which represents 100% on Figure 4(B). BV/TV values of the injured parietal bones are illustrated according to the uninjured bone. The measurments showed open bone defects and low BV/TV values in the unfilled control group (Figure 4). Increased, but not significant bone healing was seen with the uncoated grafts. In the serum albumin enhanced group we observed significantly higher BV/TV values compared with both uncoated and control groups (empty: $11.5 \pm 6\%$, uncoated: $22.5 \pm 9\%$, albumin $52.4 \pm 7\%$).

Mechanical push-out testing

Peak breaking force and material stiffness was measured in the uncoated and albumin coated group (Figure 5). Failure of the bone tissue occurred at the graft/host interface in all cases. Breaking force of the albumin coated groups was three times higher compared to the uncoated bone grafts (uncoated: 15.7 ± 4 N, albumin 46.1 ± 11 N). Similar results were found in the stiffness measurements, the albumin coated group produced significantly higher stiffness values (uncoated: 32 ± 11 N/mm^2, albumin 78.6 ± 14 N/mm^2, Figure 5).

DISCUSSION

In this study, we aimed to investigate the effects of HSA as an additive to demineralized bone in a nonhealing calvaria defect in rats. The albumin coated group healed significantly

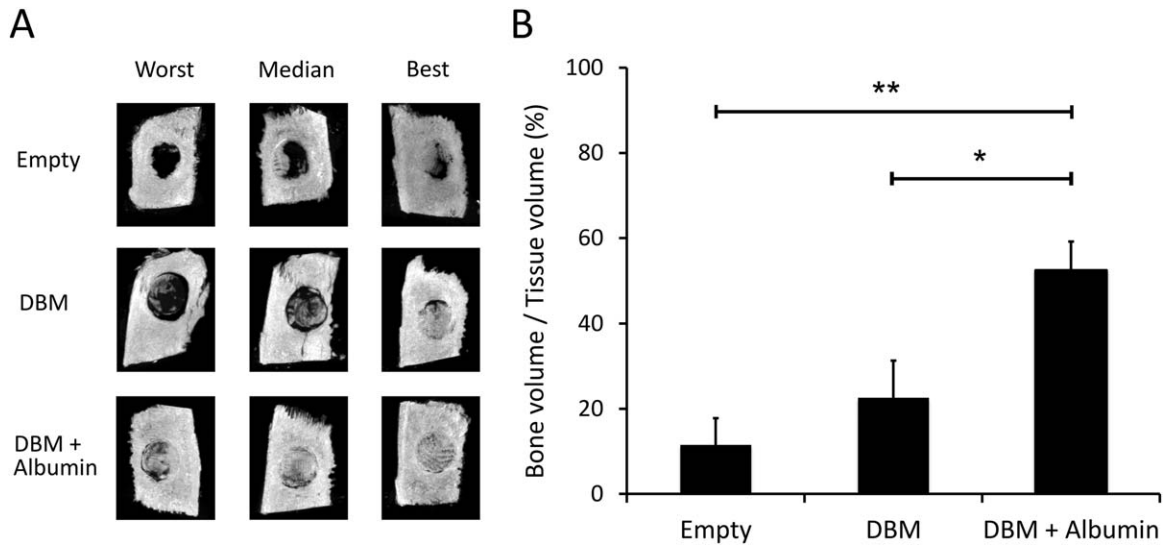

FIGURE 4. *Ex vivo* micro CT images and evaluation at 11 weeks. Panel A shows images of the harvested parietal bones in every experimental group. Panel B shows bone volume/tissue volume values in percentage. 100% represents uninjured parietal bone. DBM with albumin coating shows significantly higher BV/TV (One way ANOVA, Bonferroni posttests, *: $p < 0.05$, **: $p < 0.01$).

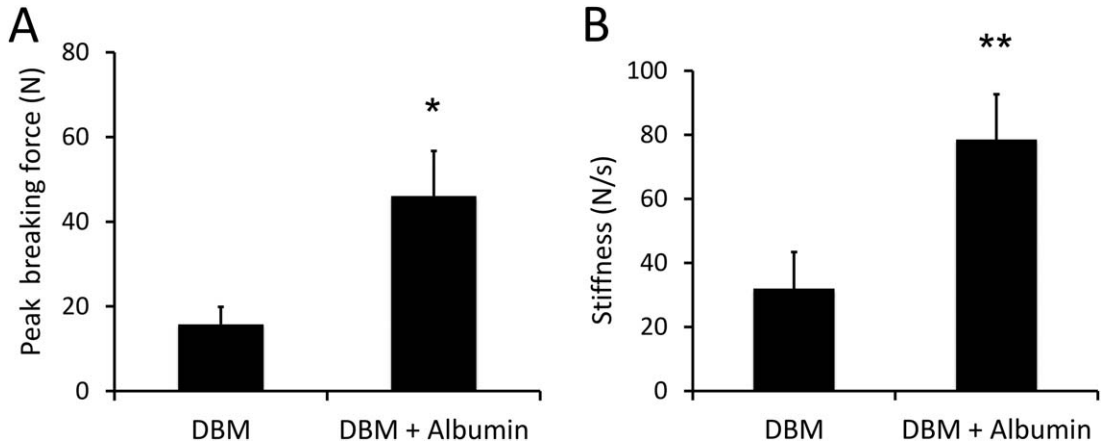

FIGURE 5. *Ex vivo* mechanical push-out testing at 11 weeks. Panel A shows peak breaking force (*N*) of the implanted material. Panel B shows the stiffness (N/mm^2) of the implanted material. Significantly higher breaking force and stiffness can be seen in the albumin coated group (Unpaired t test, *: $p < 0.05$).

faster after 3 weeks. Complete union was observed at the seventh week. In addition, the density of the remodeled bone was significantly higher in the albumin coated group. Additionally, bone specimens were tested in a mechanical testing device, which confirmed the functionally superior results in the albumin coated group.

Serum albumin is the most common protein in plasma, being responsible for numerous functions, like free radical scavenging, neutrophil adhesion and molecule transportation.[17] In addition, serum fractions like FBS, bovine serum albumin (BSA), or HSA are widely used additives in cell culture media, providing growth factors, carrying lipids, metals, and low molecular weight nutrients.[8] All of these well-described functions are possibly useful and supportive in the present experiment of bone regeneration as well. Serum albumin was also shown to reduce the colonization of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* when applied as a coating on titanium surfaces.[18–20] Serum albumin coating also reduced infectious events in a rabbit model,[21] therefore it could possibly function as a prophylactic agent against infectious complications in a variety of bone surgeries. Even though, in this present study we did not experience any surgery related infection, these data provide another beneficial mechanism of serum albumin coating in bone replacement therapies. In contrary to the anti-attachment behavior with bacteria, BSA was shown to improve adherence of osteoblast-like cells compared to adsorbed bone sialoprotein and osteopontin on hydroxyapatite surfaces.[22] The authors concluded that surface adsorbed albumin is changing the proliferation and attachment activity of the cells. Additionally, we found similar results on the surface of HSA treated surgical sutures.[23] In that experiment, the serum albumin coated sutures were able to attach significantly more MSCs after 48 h compared with classical attachment proteins like fibronectin and poly-L-lysine. More recently, data showed increased early adhesion and faster spreading of human gingival fibroblasts on BSA coated titanium surfaces.[24]. It is important to emphasize that in these, experiments serum albumin was used as a coating rather than a media supplementation, showing that serum albumin could have other favorable properties besides the above mentioned functions, like molecule transportation. In fact, Liu concluded that adsorbed BSA increased surface energy, roughness and the hydrophilicity of the coated surface, which features promoted the adherence of biological macromolecules and therefore increased the possible connecting points between cells and the material.[24] These results correlate to our present experiment very closely. We investigated cell attachment after 6, 12, and 24 h on HSA coated demineralized bone. We found that MSCs adhere to the coated surface significantly better after each time point. We believe that endogenous MSCs and precursors behave the same in our experimental model *in vivo*; thus increasing the local number of endogenous precursors at the trauma site. Numerous scientific publications are available showing that the increased number of local stem cell or precursor concentration combined with different scaffolds are potent alternatives of autologous bone replacement therapies.[25–27] The beneficial effects of increased number of local precursors are twofold. First, various stem cells are capable to differentiate into osteoblast. Second, stem cells have beneficial immunmodulatory and paracrine effects after injury as well. As a result, multiple stem cell delivery systems, scaffolds and tissue engineered bone substitutes are under intensive investigation. Interestingly, in a preliminary study Gallego showed that scaffolds fabricated from cross-linked serum albumin are suitable for osteoblast delivery. They tested their cell loaded material in a critical size defect *in vivo* and showed complete healing after 11 weeks compared with the blank control group.[28] This experiment however did not study the scaffold alone, which could supply comparative data with our outcome since we showed similar results with HSA alone.

Nonunion models always pose a limitation, as it is impossible to know for sure whether the defect will have been united at a later time-point during the lifetime of the animal. We observed that the 4 mm defect, which we used in this experiment, does not heal without treatment until

the 11th week, since there were no statistically significant reduction in the remaining bone defect of the empty bone defects from the ninth week on. This allowed a clear determination of the albumin effect as the albumin-coated group showed complete closure already. It is expected that uncoated DBM or maybe even the empty defects reach closure at a much later timepoint, however, investigating this question needs a different study design and thus falls out of the focus of the current study.

In conclusion, in this experiment nonunion bone defects were successfully treated with serum albumin coated DBM. Our grafting technique resulted in complete union and biomechanically functional new bone formation. According to the attachment pattern of MSCs, we conclude that serum albumin increases the local number of precursor cells resulting in superior bone healing. Further investigations are needed to elucidate the molecular backgrounds of serum albumin in bone regeneration, but since this protein is commonly used for other therapeutic interventions, albumin coated bone substitutes may be introduced to human clinical investigations. A case series has already been completed in implant revision surgery, which established the safety of albumin-coated bone grafts.[11] As safety in humans is established and this study showed efficacy in an animal model, a human study is justified to quantitatively assess clinical efficacy.

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