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■ **BONE BIOLOGY**

Autologous bone marrow clot as an alternative to autograft for bone defect healing

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Objectives

Long bone defects often require surgical intervention for functional restoration. The 'gold standard' treatment is autologous bone graft (ABG), usually from the patient's iliac crest. However, autograft is plagued by complications including limited supply, donor site morbidity, and the need for an additional surgery. Thus, alternative therapies are being actively investigated. Autologous bone marrow (BM) is considered as a candidate due to the presence of both endogenous reparative cells and growth factors. We aimed to compare the therapeutic potentials of autologous bone marrow aspirate (BMA) and ABG, which has not previously been done.

Methods

We compared the efficacy of coagulated autologous BMA and ABG for the repair of ulnar defects in New Zealand White rabbits. Segmental defects (14 mm) were filled with autologous clotted BM or morcellized autograft, and healing was assessed four and 12 weeks postoperatively. Harvested ulnas were subjected to radiological, micro-CT, histological, and mechanical analyses.

Results

Comparable results were obtained with autologous BMA clot and ABG, except for the quantification of new bone by micro-CT. Significantly more bone was found in the ABG-treated ulnar defects than in those treated with autologous BMA clot. This is possibly due to the remnants of necrotic autograft fragments that persisted within the healing defects at week 12 post-surgery.

Conclusion

As similar treatment outcomes were achieved by the two strategies, the preferred treatment would be one that is associated with a lower risk of complications. Hence, these results demonstrate that coagulated BMA can be considered as an alternative autogenous therapy for long bone healing.

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Article focus

- Coagulated autologous bone marrow aspirate (BMA), with its reparative properties, can contribute to segmental bone defect healing.
- Autologous BMA, with fewer known complication risks, can be considered an alternative therapy to the current standard-of-care treatment, autologous bone graft (ABG), for long bone repair.

Key messages

- A small volume of coagulated autologous bone marrow, obtained via a minimally invasive procedure, was as efficacious as morcellized autograft for long bone defect repair.

Strengths and limitations

- BMA clot represents a substitute autologous tissue for ABG that is capable of promoting bone healing.
- A limitation of this study was the exclusion of some ulnas from the data set due to larger surgically created defects. This resulted in variable sample numbers in each treatment.

Introduction

Bone marrow (BM) aspiration is a routine procedure in haematology and orthopaedics. It is conducted on either an inpatient or an outpatient basis, with complications reported to be between 0.08% and 0.12% of total procedures performed.[1-3] Both using the proper technique and minimizing peripheral blood dilution by maintaining an aspiration volume of less than 2 ml of BM per site are crucial for a successful clinical outcome.[4] The current 'gold standard' orthopaedic treatment that consistently produces successful clinical outcomes is the use of autologous bone graft (ABG). However, the acquisition of the ABG requires an additional surgical procedure, and is associated with complications such as donor site morbidity and chronic pain.[5] Dimitriou et al[6] reported that almost 20% of patients experienced complications after iliac crest bone graft harvesting. The question then arises as to whether bone marrow aspirate (BMA) alone can mitigate the shortcomings of ABG for bone repair.

The use of BM as a primary source of osteogenic progenitor cells was established by Goujon,[7] Burwell,[8] and Friedenstein et al.[9] Multipotent haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) both originate and reside in the BM, and respond to specific stimuli to migrate, proliferate, and differentiate into specialized cells.[10] For example, MSCs can differentiate into stromal cell types such as osteoblasts, adipocytes, chondrocytes, and myocytes.[11] In turn, committed cells of both progenies secrete a plethora of growth factors and cytokines that regulate cell behaviours and activities.[10] In particular, signalling factors such as bone morphogenetic proteins (BMPs) are secreted by stem cells and endothelial cells to regulate the proliferation, differentiation, and migration of various bone-forming cells.[10] Theoretically, BMA represents an attractive means of facilitating bone repair.

Although a large body of pre-clinical and clinical work has examined the effectiveness of autologous BMA to aid osteogenesis, either implanted with commercially available scaffolds[12-18] or without,[19-23] a direct comparison of the bone-healing efficacy of autologous BMA and that of the 'gold standard' ABG has not been undertaken.[24] Bone marrow aspirate alone has been introduced into injury sites by percutaneous injection,[19,20] implanted as a clot with low-level laser therapy,[23] or with ABG.[22] Heparinized autologous BMA injected percutaneously into bone repair sites has proven to be a viable treatment option in a rabbit segmental bone defect model,[19] as well as for clinical delayed unions and nonunions.[20] These, together with other successful studies employing BMA, suggest that autogenous BMA may indeed be efficacious for bone repair.[19-23]

Given the ease of use, safety, and other advantages offered by autologous BMA, we have chosen to test coagulated autogenous BMA as a standalone, point-of-care treatment. We reasoned that autologous BMA, with its osteogenic cells and osteoinductive factors, closely resembles ABG without its mineralized bone matrix. Furthermore, growth factors such as BMP-2 and other secretory proteins are released from the α-granules of platelets during marrow coagulation.[25-29] Coagulated BMA thus would seem to be a simple, easy-to-handle implant with which to harness the body's inherent regenerative potential to heal bone defects. In this study, we compared the bone-healing efficacy of autologous BMA clot and ABG in a well-established rabbit ulnar defect model.[30-34] Using a series of qualitative and quantitative analyses, we demonstrated that coagulated BMA alone offers significant advantages over ABG as the current standard-of-care for the treatment of long bone segmental defects.

Materials and Methods

Experimental design. To evaluate the bone-forming efficacy of autologous coagulated BMA, two treatment groups were employed: BMA and ABG. Defects were randomly assigned to either treatment, with 0.6 ml of BMA or ABG being used to fill each defect. Empty defects were included as negative controls. A total of 37 male New Zealand White rabbits (four to six months of age and weighing between 3 kg and 4 kg) were housed in large animal cages within a clean and conventional facility, and received food, water, and environmental enrichment *ad libitum*. Two experimental endpoints were employed: four weeks post-implantation (interim assessment) and 12 weeks post-implantation (endpoint assessment). Healing of each treated defect was evaluated by radiological, micro-CT (μ-CT), and histological analyses at both timepoints. Only the week 12 post-implantation samples were tested mechanically for functional restoration. Investigators were blinded during all data acquisition. Details of treatment assignments, sample IDs, and analysis methods are listed in Table I. All surgical procedures were performed in strict accordance with the internal guidelines issued by the Institutional Animal Care and Use Committee, A*STAR (IACUC #: 120721).

Bone marrow aspirate. Autologous BMA was aspirated using commercially available 3 ml syringes and 18-gauge needles from the iliac crest of selected New Zealand White rabbits (Table I) under general anaesthesia (75 mg/kg ketamine (Ceva, Glenorie, Australia) and 10 mg/kg xylazine (Troy Laboratories Pte. Ltd, Glendenning, Australia) injections, maintained by inhalated isoflurane) and aseptic conditions. Negative pressure was generated in the syringe by pulling back the plunger, so that marrow then entered the syringe for collection. After aspiration, a sterile Luer stop cap was added to the syringe and the aspirate was left to clot for approximately 15 minutes before implantation into a newly created ulnar defect of the same animal.

Table I. Study rabbit ulna sample list showing sample IDs with associated analyses performed

Treatment / sample ID	Radiograph	μCT	H&E	RT	Torsional
Week 4					
Y369R Left	Yes	No	Yes	N/A	N/A
Y386R Left	Yes	No	Yes	N/A	N/A
W750L Right	Yes	Yes	Yes	N/A	N/A
W751L Left	Yes	Yes	Yes	N/A	N/A
Blk757L Left	Yes	Yes	Yes	N/A	N/A
Y958L Left	Yes	Yes	Yes	N/A	N/A
ABG					
R181L Left	Yes	Yes	Yes	N/A	N/A
R182L Right	Yes	Yes	Yes	N/A	N/A
W736L Right	Yes	Yes	Yes	N/A	N/A
W750L Left	Yes	Yes	Yes	N/A	N/A
W752L Left	Yes	Yes	Yes	N/A	N/A
Blk752L Right	Yes	Yes	Yes	N/A	N/A
R176R Left*	Yes	Yes	Yes	N/A	N/A
W737L Left*	Yes	Yes	Yes	N/A	N/A
BMA					
Y823L Left	Yes	Yes	Yes	N/A	N/A
W807R Left	Yes	Yes	Yes	N/A	N/A
Y755L Right	Yes	Yes	Yes	N/A	N/A
Y767L Right	Yes	Yes	Yes	N/A	N/A
W782L Right*	Yes	Yes	Yes	N/A	N/A
Y767L Left*	Yes	Yes	Yes	N/A	N/A
Week 12					
Blk727L Right	Yes	Yes	Yes	Yes	No
Blk735R Right	Yes	Yes	Yes	Yes	No
ABG					
Blk733R Left	Yes	Yes	No	No	Yes
Blk725L Left	Yes	Yes	No	No	Yes
Blk735R Left	Yes	Yes	No	No	Yes
Y944L Right	Yes	Yes	No	No	Yes
Y947L Right	Yes	Yes	No	No	Yes
Y391R Right	Yes	Yes	Yes	Yes	No
W726R Left	Yes	Yes	Yes	Yes	No
Blk721L Right	Yes	Yes	Yes	Yes	No
Y371R Right*	Yes	Yes	Yes	Yes	No
BMA					
Y808L Right	Yes	Yes	Yes	Yes	Yes
Blk544L Left	Yes	Yes	Yes	No	Yes
Blk578L Left	Yes	Yes	Yes	No	Yes
Y817L Right	Yes	Yes	Yes	Yes	Yes
Y757R Right	Yes	Yes	Yes	Yes	Yes
Y765L Right	Yes	Yes	Yes	Yes	Yes
Y756L Left	Yes	Yes	Yes	No	Yes
Y786L Right	Yes	Yes	Yes	Yes	No†

*Excluded sample due to defect size >16.1 mm
†Excluded sample due to breakage before mechanical testing
H&E, haematoxylin and eosin; RT, ralis tetrachrome; N/A, not applicable; ABG, autologous bone graft; BMA, bone marrow aspirate

Surgical implantation. Bilateral mid-diaphyseal ulnar defects (14 mm in length) were created and treatments were randomly assigned as previously described.[32,33] The ulna was measured, and its midpoint was identified and marked using a surgical marker with ruler set (Devon; Cardinal Health Inc., Dublin, Ohio). A central 6 cm skin and muscle incision was made, and the periosteum on a centralized 15 mm bone segment was removed. The midpoint was again identified and the two bony cut-ends measuring 14 mm across were marked. An Acculan miniature bone saw (Braun Mesungen, Hessen, Germany) was then used to create the defect, sparing the underlying radius and its periosteum. For animals receiving ABG (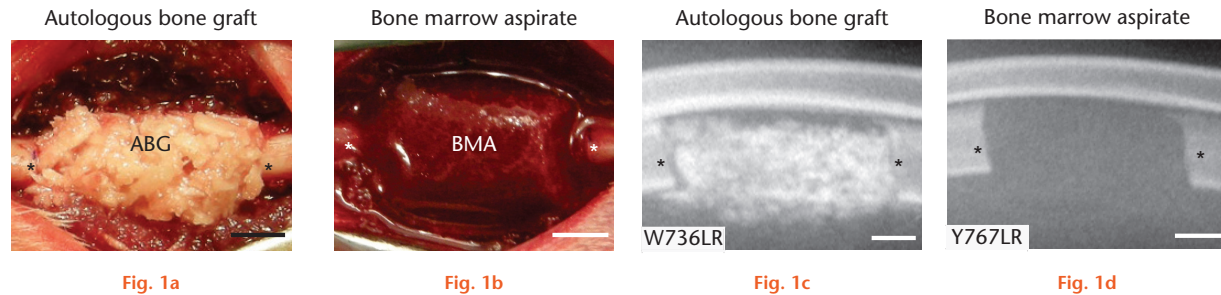Table I), the resected ulna segment containing both cortical and cancellous bone was morcellized using a micro-bone mill (Aesculap; Braun) and 0.6 ml of fragments were then implanted back into the defect. For the BMA group (Table I), the autologous BMA clot was released from the syringe and implanted directly into the bone defect. Wounds were then closed with muscular and skin stitches, and Vetbond tissue adhesive (3M, St. Paul, Minnesota) was applied. All animals were given antibiotics (Baytril; Bayer, Leverkusen, Germany; 10 mg/kg, once a day) and analgesic (buprenorphine, 1 mg/kg, twice a day) subcutaneously for five days post-surgery and were left to heal until their respective experiment endpoints. All rabbits healed uneventfully, without any sign of infection or illness. The number of operated

Table II. Scoring criteria for bone formation[28] for each region within ulnar defect

Score	Extent of bone formation
0	No bone formation
1	Less than 25% bone formation
2	Less than 50% bone formation
3	Less than 75% bone formation

Table III. The International Society of Limb Salvage (ISOLS) Radiological Implant Evaluation System[29] for bone fusion assessment

Score	Extent of bone fusion
1	No callus formation or fusion of less than 25% of cortical thickness
2	25% to 75% bone fusion of cortical thickness
3	More than or equal to 75% cortical thickness with a visible osteotomy line
4	100% fusion with no visible osteotomy line

Autologous bone graft — Bone marrow aspirate — Autologous bone graft — Bone marrow aspirate

ABG | BMA | W736LR | Y767LR

Fig. 1a — Fig. 1b — Fig. 1c — Fig. 1d

Implantation of autologous bone graft (ABG) and bone marrow aspirate (BMA) treatments into ulnar defects. Representative a) and b) post-implantation digital images, and c) and d) post-surgical radiographs of ABG and BMA treatments in rabbit ulnar defects. Scale bar indicates 3.5 mm. *Osteotomized bone end.

defects in the empty, ABG, and BMA groups were n=6, n=8, and n=6 for week 4, respectively, and n=2, n=9, and n=8 for the week 12 timepoint, respectively.

Radiological and μ-CT analyses. To evaluate bone healing, radiographs (MUX-100; Shimadzu, Tokyo, Japan) were taken immediately post-surgery and again at four and 12 weeks post-treatment, as described previously.[32,33] Radiographs taken immediately post-surgery were used to confirm a defect size of 14 mm in length. An allowance of 15% of defect length (11.9 mm to 16.1 mm; SD 2 mm for the combined thickness of the bone saw blade) was used for sample inclusion, and defects meeting these criteria underwent further analyses.

Endpoint radiographs were scored by three independent reviewers (including ZXHL and TCT), who were blinded to the treatment groups, to assess the extent of bone formation and fusion of the defects. For bone formation, each defect was scored between 0 and 3 at each of four sites, incorporating the proximal, middle, and distal regions of the defect and the interface between the radius and the ulna, as previously described (Table II).[33] For bone fusion assessment, the International Society of Limb Salvage (ISOLS) Radiological Implant Evaluation System was used.[35] Each defect was scored for bone fusion across its cortical thickness (Table III). Therefore, a maximum score of 12 was possible for robust bone formation, and of 4 for radiological fusion assessments.

Additional assessment of bone healing was obtained by μ-CT analysis (Skyscan 1076; Skyscan, Aartselaar, Belgium) of harvested limbs, as previously reported.[32,33] Following 3D reconstruction (Mimics; Materialise, Leuven, Belgium), quantification of bone formation was determined at both timepoints, as bone volume within the ulnar defect, excluding the radius (mm³).

Mechanical testing. Torsional testing was performed on five of eight randomly selected ABG samples and seven of eight BMA samples at 12 weeks post-implantation. One of the BMA samples broke during sample preparation prior to mechanical testing and was excluded (Table I).

Sample preparation was performed according to our previous publications.[32,33] For the ABG group, the embedded samples were mounted onto an MTS 858 Mini Bionix II testing system (MTS Systems Corp., Eden Prairie, Minnesota) and tested until failure at a constant angular loading rate of 1° per second. Similarly for the BMA group, the embedded samples were mounted on an MTS Bionix Electromechanical Torsion test system (MTS Systems Corp., Eden Prairie, Minnesota) and tested until failure at a constant angular loading rate of 1° per second. Both test systems were validated prior to experimental sample testing to ensure repeatability of results. Analyzed BMA samples were then kept in phosphate-buffered saline (PBS), followed by histological sample preparation. For each sample, the torque *versus* angular displacement curve was plotted and the torsional stiffness, represented by the gradient of the linear portion of the graph, was derived and normalized against the diameter of the healed ulna. Additionally, maximum torque and angle at failure were also recorded for each sample.

Histological analyses. Histology was performed on all samples from the BMA group at both timepoints after mechanical testing to maximize output from the data set. For the ABG group, samples that did not undergo mechanical testing were sent for histological preparation and evaluation.

Histological preparation was performed for the aforementioned samples as per our previous publications.[32,33] The histology sections were then stained with haematoxylin
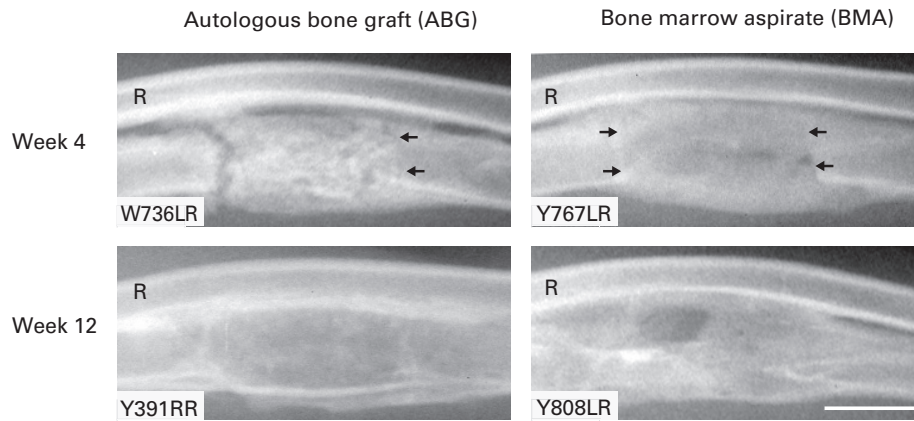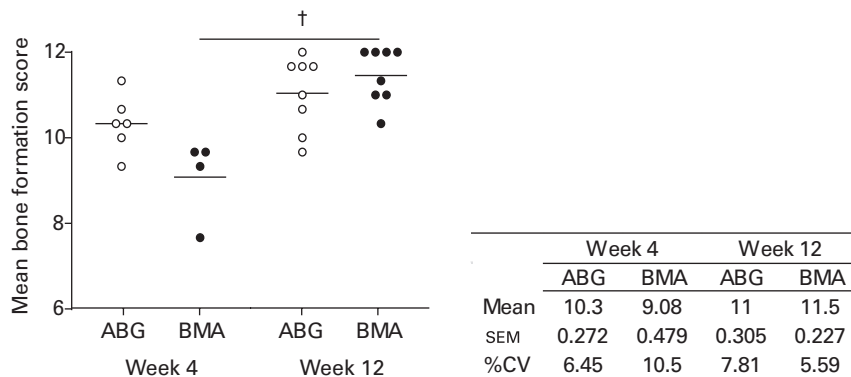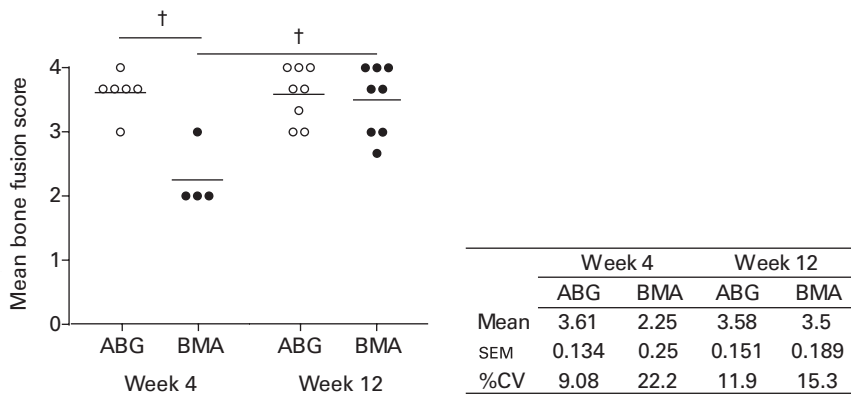
Fig. 2a

	Week 4		Week 12	
	ABG	BMA	ABG	BMA
Mean	10.3	9.08	11	11.5
SEM	0.272	0.479	0.305	0.227
%CV	6.45	10.5	7.81	5.59

Fig. 2b

	Week 4		Week 12	
	ABG	BMA	ABG	BMA
Mean	3.61	2.25	3.58	3.5
SEM	0.134	0.25	0.151	0.189
%CV	9.08	22.2	11.9	15.3

Fig. 2c

Radiological assessment of rabbit ulnar defects at four and 12 weeks post-surgery. Radiographs were taken of ulnas after animal sacrifice for evaluation of bone deposition. a) Representative radiographs of autologous bone graft (ABG)-treated and bone marrow aspirate (BMA)-treated ulnar defects at both timepoints. Arrows indicate osteotomy lines. Scale bar indicates 7 mm. R, radius. Three independent reviewers (including ZXHL and TCT) scored endpoint radiographs for b) bone formation and c) bone fusion within each treated defect. The average score of each sample was assessed with scatter plots, with the means, standard errors of the means (SEMs), and coefficients of variation (%CV) shown in tables adjacent to each plot. †p < 0.001.

and eosin (H&E) for general morphology, as previously described.[33,36,37] Additionally, sections from week 12 post-surgical samples were stained with modified ralis tetrachrome (RT)[38] for bone mineralization. Thus, the number of ulnar samples in the empty, ABG, and BMA groups that underwent modified RT staining were n=2, n=3, and n=5, respectively, for the later timepoint.

Statistical analysis. Data were reported as means ± standard errors of the means (SEMs). All results were analyzed using two-way analysis of variance (ANOVA) with Tukey *post hoc* testing, except for the biomechanical data that were analyzed using a Student's *t*-test (Graphpad Prism; GraphPad Software Inc., La Jolla, California); p-values <0.05 were considered significant.

Table IV. Interobserver errors for bone formation and fusion scoring

Score	Week 4		Week 12	
	ABG	BMA	ABG	BMA
Bone formation, range of SEMs	0.000 to 0.333	0.000 to 0.577	0.000 to 0.667	0.000 to 0.577
Bone fusion, range of SEMs	0.333 to 0.667	0.333 to 1.200	0.000 to 0.667	0.000 to 0.882

ABG, autologous bone graft; BMA, bone marrow aspirate

Results

Surgery and treatment implantation. A 14 mm mid-diaphyseal segment of each ulna was extracted, leaving behind an empty defect (Supplementary Figs aa and ab). For ABG treatment, this resected bone segment was morcellized, and approximately 0.6 ml of the ABG fragments was used to fill the newly created defect (Figs 1a and 1c). For BMA treatment, the autologous marrow clot was released from the syringe and implanted into the ulnar defect (Figs 1b and 1d).

When measuring the lengths of newly created ulnar defects from post-surgical radiographs, five samples had defects that failed to meet the inclusion criteria and were excluded from the study (Table I). No defects measuring less than 14 mm were detected. Therefore, at week 4, analyses were performed on empty (n=6), ABG (n=6), and BMA (n=4) groups, and at week 12, analyses were performed on empty (n=2), ABG (n=8), and BMA (n=8) groups (Table I).

Qualitative radiological assessment of bone formation. Radiographs were taken of harvested ulnas at four weeks and 12 weeks post-surgery. At four weeks, morcellized bone fragments filled the defects treated with ABG, and the osteotomy lines were still visible (Fig. 2a). For the BMA group, the osteotomy lines also remained visible, with incomplete bone filling and callus formation evident. Also, the newly formed bone was less radio-opaque than the host bone. By 12 weeks post-implantation, ABG-treated defects showed complete bridging and fusion, and the osteotomy lines were invisible. For BMA-treated defects, bone bridging was observed between the two ends of the defect, although bone filling within some defects was incomplete. Empty defects did not completely fill with new bone for either timepoint; instead, areas devoid of bone were observed to span the lengths of the defects (Supplementary Figs ac and cd).

Additionally, three independent reviewers scored the radiographs to assess bone formation and fusion of defects. With a maximum score of 12, the mean bone formation scores of ABG-treated and BMA-treated ulnas were 10.3 (SEM 0.272) and 9.08 (SEM 0.479) at week 4, and 11 (SEM 0.305) and 11.5 (SEM 0.227) at week 12 post-surgery, respectively (Fig. 2b). No significant difference was found between the treatments at either timepoints. However, the mean bone formation score increased significantly from 9.08 at week 4 to 11.5 at week 12 post-surgery for BMA (p<0.001). Furthermore, the coefficients of variance (CV) for ABG and BMA treatments at the early timepoint were 6.45% and 10.5%, and 7.81% and 5.59% at the later timepoint, respectively, reflecting low variabilities in both treatment outcomes. Lastly, the interobserver SEMs of all treatment groups at both timepoints were found to be below 0.6, with the exception of ABG at week 4 post-implantation being 0.667 (Table IV).

For bone fusion, similar mean scores were observed for ABG and BMA treatments at week 12 post-surgery (3.58 (SEM 0.151) and 3.5 (SEM 0.189), respectively) (Fig. 2c). However, at week 4, fusion was significantly higher (p<0.001) following ABG treatment (3.61 (SEM 0.134)) compared with BMA (2.25 (SEM 0.25)). Also, fusion scores for BMA-treated defects significantly increased (p=0.001) over time, whereas time had no effect on fusion scores for ABG. Instead, both bone fusion and formation scores for ABG treatment reached their maximums by week 4 (Figs 2b and 2c). Additionally, the variabilities in fusion scores were higher for BMA treatment than for ABG at the early timepoint (CV of 22.2% and 9.08%, respectively), but were similar by the later timepoint (CV for BMA = 15.3%; CV for ABG = 11.9%). Lastly, the interobserver SEMs of the ABG group at both timepoints were found to be below 0.667, while those of BMA decreased from 1.2 at week 4 to 0.882 at week 12 post-implantation (Table IV).

Quantification of bone deposition by μ-CT. Having observed similar healing responses across both treatments, we next sought to quantify the volume of bone formed within the treated defects by μ-CT. After four weeks of healing, the defects treated with ABG appeared highly irregular (Fig. 3a). By 12 weeks post-surgery, the contours of the ABG-treated defects appeared more uniform. Despite these differences, the mean volumes of bone found within the ABG-treated defects were similar across the two timepoints (183.9 mm^3 (SEM 16.4) at week 4; 185.1 mm^3 (SEM 13.8) at week 12; Fig. 3b). For BMA-treated defects (Fig. 3b), the amount of new bone within the defect increased significantly over time (68 mm^3 (SEM 16.9) at week 4; 135.7 mm^3 (SEM 10.1) at week 12; p=0.024), yet this was significantly less than that of the ABG treatment at both timepoints (week 4, p<0.001; week 12, p=0.0499). This was to be expected given the persistence of morcellized bone graft material, which contributed to the higher bone volume measurements (Figs 4a and 4b; Supplementary Fig. b).

Next, we examined bone healing in three regions (zone 1, proximal; zone 2, middle; zone 3, distal) along the length of the ulnar defects. At neither timepoint was any significant difference in bone volume observed among the three regions for either treatment (Supplementary Fig. c). No bone was observed in the centre of the 3D reconstructed models of the empty defects for either timepoint (Supplementary Figs ac and ad).
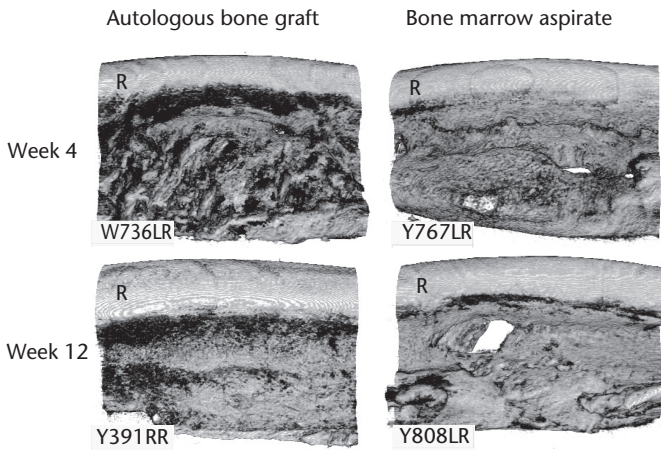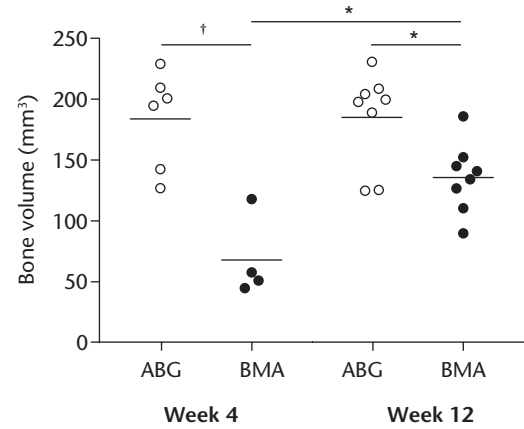
Fig. 3a

Fig. 3b

Micro-CT assessment of rabbit ulnar defects at four and 12 weeks post-surgery. a) Representative 3D models reconstructed by Mimics software (Materialise, Leuven, Belgium). R, radius. b) Bone quantified within the 14 mm autologous bone graft (ABG)-treated and bone marrow aspirate (BMA)-treated defects (mm³). *p<0.05; †p<0.001.
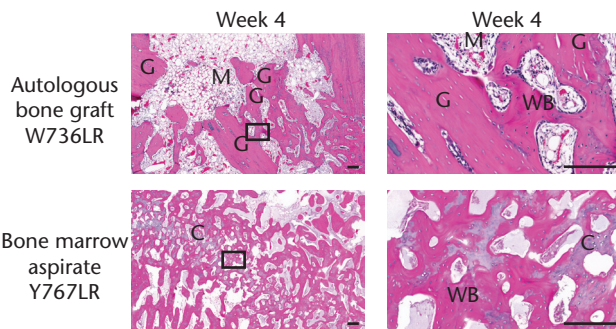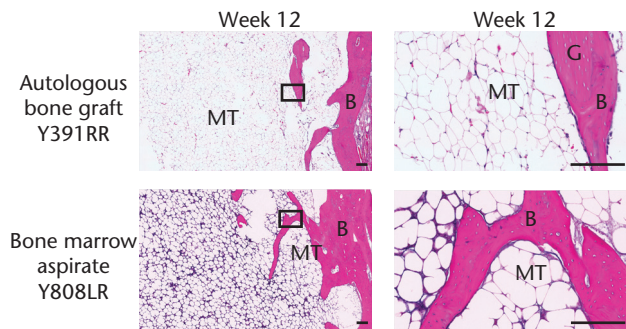

Fig. 4a

Fig. 4b

Bone healing assessments by histology. Representative images of haematoxylin and eosin (H&E)-stained sections taken from samples approximating the mean bone volume scores from the μ-CT analyses. Images show general morphology of healing bone defects at a) post-surgical week 4 and b) week 12. Images in the right-hand column are high-power magnifications of the boxed areas in the low magnification images on the left. Scale bar represents 200 μm. G, autologous graft; M, marrow; WB, woven bone; C, chondrocytes; B, bone; MT, marrow-like tissue.

Histological assessment of bone formation. Histology was performed on harvested samples to assess the general morphology and the extent of bone mineralization within the healing defects. At week 4 post-implantation, numerous autograft fragments with empty osteocytic lacunae among woven bone and marrow were still visible (Fig. 4a; Supplementary Fig. ba). Additionally, remodelling cavities were observed that were lined with osteoclasts and osteoblasts (Supplementary Fig. ba). In contrast, newly formed woven bone with some hypertrophic chondrocytes and calcified matrix were observed in the BMA-treated ulna (Fig. 4a). Osteoblasts and osteoclasts were also observed in defects treated with BMA (Supplementary Fig. ba).

By week 12 post-treatment, mineralized lamellar-like bone staining blue/red and marrow-like tissue were observed in ABG-treated ulnas (Supplementary Fig. d). Moreover, persistent remnants of autograft with empty lacunae were seen encased in new bone (Fig. 4b; Supplementary Fig. bb). For BMA, mineralizing lamellar-like bone with marrow-like tissue was found in the defects (Fig. 4b; Supplementary Fig. d). For both groups, osteoblasts were observed to line remodelling cavities, with osteoclasts also present (Supplementary Fig. bb).

Notably, for untreated defects (empty control), soft tissue was observed among areas of new bone at week 4 and week 12 post-surgery (Supplementary Figs ac and ad).

Assessment of mechanical strength by torsional testing. Functional restoration was examined by determining how much rotational force each treated ulna could withstand before failure, or to a maximum of 35° for ABG or 50° for BMA, whichever occurred first. At week 12 post-implantation, the mean normalized stiffness of ABG, BMA, and intact bone were 0.004715 Nm/°mm, 0.007616 Nm/°mm, and 0.01065 Nm/°mm, respectively (Fig. 5a). When compared with the stiffest sample in the ABG group with 0.006 Nm/°mm, four out of eight BMA-treated samples (57%) were found to be stiffer.
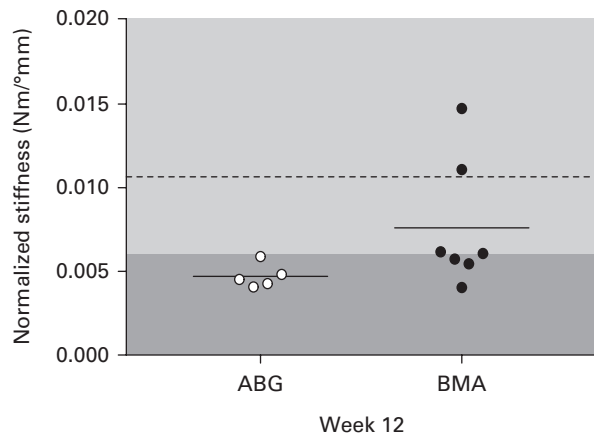
Fig. 5a

Fig. 5b

Normalized stiffness (Nm/°mm)	ABG	BMA
> 0.006	0/5 (*0*)	4/7 (*57*)
0 to 0.006	5/5 (100)	3/7 (*43*)
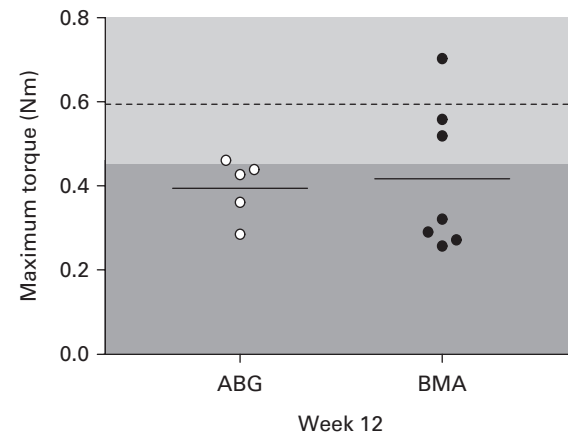
Maximum torque (Nm)	ABG	BMA
> 0.46	0/5 (*0*)	4/7 (*57*)
0 to 0.46	5/5 (100)	3/7 (*43*)

Torsion testing of week 12 post-implantation samples. a) Stiffness was normalized against individual ulna diameter (nm/°mm), and b) against maximum torque (nm) of treated defects. Solid lines indicate the mean values of samples while dotted lines indicate mean values of reference (intact) bones. The numbers and the associated percentages of bone marrow aspirate (BMA)-treated samples with higher normalized stiffness and maximum torques than the maximum autologous bone graft (ABG)-treated ulnas were determined.

Additionally, two out of eight BMA-treated ulnas (25%) had greater stiffness than the uninjured ulnas, while none of the ABG-treated ulnas outperformed uninjured bone for normalized stiffness.

At maximum torque, the mean values for ABG, BMA, and intact bone were 0.3946 nm, 0.417 nm, and 0.5958 nm, respectively (Fig. 5b). Only one BMA-treated sample performed better than intact bone in this parameter, while all of the ABG-treated ulnas had lower maximum torque than that of intact bone. When compared with the sample with the highest maximum torque in the ABG group (0.46 nm), three out of seven of the BMA-treated samples (43%) outperformed this data point.

Discussion

Autograft harvested from a patient's iliac crest constitutes the current standard of care for segmental bone loss in orthopaedic surgery.[39] Autologous bone is an ideal graft because it contains both osteogenic cells and osteoinductive factors contained within an osteoconductive matrix.[40] Despite its clinical success, its use is still limited by complications such as associated donor site morbidity and pain.[5,6] We propose the use of another tissue, coagulated autologous BMA, as an alternative therapy for bone repair. Bone marrow aspirate is a rich source of osteoinductive factors produced by resident cells such as MSCs, endothelial cells, osteoblasts, macrophages, and platelets.[10,28,29,41-43] It is essentially an autograft without the bone matrix, and its healing potential is beginning to gain clinical support. For example, percutaneous BMA injections for delayed unions or nonunions resulted in long bone bridging in more than 70% of patients.[20,44] To

this end, we demonstrated that long bone repair via the implantation of autogenous BMA clot was as successful as ABG treatment in a rabbit ulnar defect model.

We evaluated the bone repair efficacy of ABG by implanting morcellized corticocancellous ulnar bone that was extracted during defect creation. At week 4 post-implantation, osteotomy lines were visible and woven bone was observed among remnant autograft fragments with their chacteristic empty osteocytic lacunae. These suggest the start of bone remodelling to unify the bone ends and replacement of ABG fragments with new immature bone. By week 12 post-implantation, smooth, fully bridged defects were filled with mineralized bone with some persistent autograft fragments. This suggests extensive remodelling of old bone fragments and matured bone. However, mechanical restoration of ABG-treated ulnas never approached that of uninjured bone. This observation was similar to those reported for cortical bone graft incorporation.[45-47] After graft implantation, the formation of a local haematoma is followed by inflammation. We suggest that creeping substitution[47] then occurs where the majority of the cortical autograft will undergo necrosis[45] and be resorbed by osteoclasts, while new bone is deposited by osteoblasts via intramembranous ossification. However, as graft resorption is often incomplete, the resultant mixture of autograft remnants and viable new bone leads to mechanical weakness.[46]

In comparison with ABG, with its associated complications, all rabbits in this study tolerated the BM aspiration procedure well, with no observed post-procedure complication. This corroborates the current clinical concensus

that BMA is a safe procedure, with complications being encountered only rarely.[48] It was important to establish negative pressure in the syringe before aspiration, as this indicated the correct positioning of the needle into the rabbit iliac crest intramedullary cavity.[4] Moreover, aspiration volumes were kept to 0.6 ml of BMA per site to avoid dilution with peripheral blood, reportedly a key contributor to poor clinical outcomes following BMA procedures.[4] Importantly, the aspirated autologous tissue was allowed to clot in order to generate the implantable material that has improved handling properties over percutaneous injections of heparinized marrow[19,20] or commercially available synthetic bone graft material such as Healos (DePuy Synthes, Raynham, Massachusetts)[13,17] and Vitoss (Stryker, Kalamazoo, Michigan)[18] infused with BMA. Furthermore, activated platelets will degranulate, releasing pro-osteogenic, pro-angiogenic, and pro-mitogenic growth factors such as BMP-2, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF-2) during BMA clotting.[25-29] Therefore, the defect site would contain reparative cells and active growth factors trapped within the implanted marrow clot, as well as in the haematoma formed as a result of the injury. Indeed, Shoji et al[49] found significantly higher levels of growth factors in BMA clot compared with peripheral blood clot. In turn, a heightened chemotactic response of reparative cells such as MSCs[50] may be present at the defect site. Furthermore, animal studies have shown better bone healing with autologous BMA than with peripheral blood.[16,22,23] Thus, this combination of intrinsic repair components from autologous BMA and haematoma may enhance bone healing.

In support of this hypothesis, woven bone with osteoblastic remodelling cavities, as well as hypertrophic chondrocytes and extracellular matrix (ECM), were found to have partially filled BMA-treated defects at week 4 post-implantation. It is known that pro-osteogenic and pro-angiogenic factors in the cartilaginous ECM produced by chondrocytes during endochondral ossification[51] result in mineralization and woven bone formation.[52] This suggests that bone repair in the BMA group occurred through both endochondral and intramembranous ossification. By week 12 post-implantation, mineralizing bone filled the BMA-treated defects and the extent of bone fusion in both BMA- and ABG-treated ulnas was similar. Functionally, approximately 50% of the BMA-treated defects had mechanical strength measurements that outperformed those of ABG. Collectively, these results suggest that coagulated BMA is as effective as ABG for long bone defect healing. This is in agreement with the notion that therapeutic efficacy in bone healing depends largely on osteogenic cells and osteoinductive growth factors, which are present in both autograft and BMA.[21] Mineralized bone matrix is thought to play an auxiliary role. It is worth noting that the BMA healing process does not involve the

resorption of bone graft fragments that may prolong the reparative cascade. Indeed, autologous BMA has been shown to be an adjuvant to ABG in augmenting spinal arthrodesis in a rabbit spinal fusion model.[22] This synergistic bone repair effect is based on intrinsic healing components of both autologous tissues. As rabbit and human bone share similar toughness and mineral densities,[53] it is plausible that they share the same healing mechanisms.

A limitation of this study was the exclusion of some ulnas from the data set due to the surgical creation of larger defects. This led us to impose study exclusion criteria that had an upper limit of 15% above the ideal 14 mm defect size; any defects that measured more than 16.1 mm from radiographs taken immediately post-operation were excluded. The larger defect size would have meant a larger healing demand due to an increase in the defect volume. These exclusion criteria contributed to the variable sample numbers in each treatment. Another limitation was the imperfect alignment of the bone ends during embedding for mechanical testing. This possibly resulted in a shift of the sample's gravity axis relative to the rotational axis of the torsional testing setup, leading to a small experimental error in the sample's measured stiffness. Steiner et al[54] demonstrated a correlation between the parallel offset of the torsional axis and the resultant error in stiffness measured. They also noted that the perfect conditions described are hard to achieve experimentally. Lastly, the autograft used here was the resected ulnar bone, which was then morcellized before implantation. This meant that predominantly cortical bone fragments with some cancellous bone were implanted into the defect site. We did not test other sources of autografts such as the iliac crest, fibula, or tibia, which may have different cortical/cancellous bone compositions when compared with the ulna. Additionally, these autografts are each associated with various degrees of donor site morbidity,[55] which may result in higher or lower complication risks.

Several pre-clinical and clinical studies have utilized autogenous BM for bone healing with varied results. In this study, a small volume of clotted autologous BM obtained via a minimally invasive procedure was found to be equally as efficacious as ABG for long bone repair. Further validation of the clinical efficacy of this native material is clearly warranted.

Supplementary material

ë Additional figures showing healing quantity and quality following treatment of ulna defects at four and 12 weeks post-surgery. A minimal healing response was observed for untreated ulna defects at both timepoints.

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