Cytotherapy, 2013; 15: 242–252

International Society for Cellular Therapy
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Enhancing the osteogenic efficacy of human bone marrow aspirate: concentrating osteoprogenitors using wave-assisted filtration

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Abstract

Background. Recent approaches have sought to harness the potential of stem cells to regenerate bone that is lost as a consequence of trauma or disease. Bone marrow aspirate (BMA) provides an autologous source of osteoprogenitors for such applications. However, previous studies indicated that the concentration of osteoprogenitors present in BMA is less than required for robust bone regeneration. We provide further evidence for the importance of BMA enrichment for skeletal tissue engineering strategies using a novel acoustic wave-facilitated filtration strategy to concentrate BMA for osteoprogenitors, clinically applicable for intraoperative orthopedic use. *Methods.* Femoral BMA from 15 patients of an elderly cohort was concentrated for the nucleated cell fraction against erythrocytes and excess plasma volume via size exclusion filtration facilitated by acoustic agitation. The effect of aspirate concentration was assessed by assays for colony formation, flow cytometry, multilineage differentiation and scaffold seeding efficiency. *Results.* BMA was filtered to achieve a mean 4.2-fold reduction in volume with a corresponding enrichment of viable and functional osteoprogenitors, indicated by flow cytometry and assays for colony formation. Enhanced osteogenic and chondrogenic differentiation was observed using concentrated aspirate and enhanced cell-seeding efficiency onto allogeneic bone graft as an effect of osteoprogenitor concentration relative specifically to the concentration of erythrocytes in the aspirate. *Conclusions.* These studies provide evidence for the importance of BMA nucleated cell concentration for both cell differentiation and cell seeding efficiency and demonstrate the potential of this approach for intraoperative application to enhance bone healing.

Key Words: *bone marrow aspirate, bone marrow stem cells, bone regeneration, colony-forming unit-fibroblastoid, mesenchymal stem cells, osteoprogenitors, tissue engineering*

Introduction

Tissue engineering strategies seek to harness the regenerative potential of stem or progenitor cells to replace tissue lost or damaged through injury or disease. In orthopedics, applications may include arthritides, spinal fusion, tendinopathies and bone stock replacement after trauma, after excision of neoplastic or infected bone, or for revision arthroplasty (1–3). Aspirated bone marrow possesses considerable potential in such applications as an autologous source of osteoprogenitors able to regenerate bone and cartilage tissue. Although connective tissue progenitors are resident in many tissues, bone marrow serves as the richest and most readily available repository of progenitor cells capable of differentiating into mature bone-forming cells (4); much of the osteogenic capacity of autologous bone graft derives from the bone marrow component (5).

However, despite some notable isolated successes in which the regenerative capacity of autologous bone marrow aspirate (BMA) has been successfully applied in the treatment of chronic wounds (6) and in combination with demineralized bone matrix for the treatment of aneurysmal (7) and unicameral (8) bone cysts, variable results obtained with aspirated cells suggest that concentration of BMA for osteoprogenitor cells may be required to sustain robust skeletal regeneration (9–12). The proportion of osteoprogenitors present in bone marrow is typically only approximately 0.005% of total nucleated cells (NCs), and these numbers are variable, dependent

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(Received 3 July 2012; accepted 10 September 2012)

http://dx.doi.org/10.1016/j.jcyt.2012.09.004

on sampling technique, volume and site (13–16). Although this low cell concentration is sufficient when transplanting bulk autograft bone, where cells are translocated within their existing osteoinductive environment (and supplemented by osteoprogenitors present within the bone matrix (17,18)), robust skeletal regeneration using aspirated bone marrow appears likely to require pre-emptive cellular enrichment (19–21).

Key clinical and pre-clinical studies have established the importance of osteoprogenitor concentration and number on bone repair. Early clinical and pre-clinical studies by Connolly *et al.* (10,22) indicated that the NC concentration of BMA was significant for bone formation and repair. More recently, Hernigou *et al.* (23) successfully treated 53 of 60 patients with femoral head osteonecrosis or fracture non-unions via percutaneous injection of BMA concentrated for osteoprogenitors. Retrospective analysis of osteoprogenitor number (by colony-forming unit-fibroblastoid [CFU-F] analysis) revealed that the seven atrophic non-unions that failed to heal had significantly lower numbers and concentration of osteoprogenitors.

In the current study, we demonstrate the potential to concentrate, via filtration, the nucleated fraction of bone marrow aspirate obtained from a clinically relevant elderly cohort and provide *in vitro* evidence that may account for the significance osteoprogenitor concentration possesses for the regenerative efficacy of BMA. Adopting an approach to cell filtration applicable within the sterile field of an operating room (24), we demonstrate the ability to process and enrich BMA efficiently for osteoprogenitors and the significance of cell concentration for osteogenic and chondrogenic cell differentiation and seeding efficiency onto allogeneic bone graft.

Methods

Patient selection

Volunteers were selected from hematologically normal patients undergoing primary total hip replacement for symptomatic osteoarthritis. Exclusion criteria were pre-existing conditions that may have confounding effects on cellular activity, including a history of Paget's disease of bone, malignancy, clotting disorders, osteogenesis imperfecta, rheumatoid arthritis, osteonecrosis of the femoral head, long-term bisphosphonate or glucocorticoid therapy and known transmissible disease (e.g., hepatitis, human immunodeficiency virus, malaria) or sickle cell disease. Patients provided fully informed consent after receiving a lay summary of the trial, together with a verbal explanation and an opportunity for questioning. Ethical approval of the study was obtained locally (LREC194/99/1) and regionally (Research Ethics Committee 09/H0505/5). MHRA (Medicines and Healthcare products Regulatory Agency) registration was unnecessary at this stage because the device was not being used for direct therapeutic intervention.

Bone marrow harvest from the femoral canal

BMA for *in vitro* experimentation was harvested as waste tissue from the femoral canal during total hip replacement surgery. Before aspiration of bone marrow, a 20 mL Luer-Lok syringe containing 5000 IU of heparin in 5 mL of normal saline was prepared and the needle was flushed with heparin. Bone marrow was aspirated from a location proximal to the pending neck osteotomy into the heparinized syringe and transferred to a sterile universal container for transport from the operating room to laboratory for filtration.

Operation of the device

BMA was concentrated via a vacuum-assisted filtration approach facilitated by acoustic agitation (Figure 1A–C). Filters were manufactured from 23 μm-thick polyethylene terephthalate to achieve a range of uniform pore sizes (1.2–4.1 μm diameter) and porosities (150 000–1.5 million pores/cm^2) via a track etching process (it4ip, Seneffe, Belgium). Acoustic vibration was applied using a voice-coil (model RM-ETNC0033K19C-2KOI; NXT Technology, Cambridge, UK). Vacuum pressure was produced with a 6V miniature diaphragm pump (KPV14A-6A; Koge Electronics Co., Ltd, Taipei, Taiwan).

Following straining through a 70 μm pore-size pre-filter (Figure 1B, ii), each BMA sample was applied to a size-exclusion filter positioned above a reservoir of phosphate-buffered saline (PBS) (Figure 1B, iii). A resonant frequency wave was passed through the PBS reservoir, agitating the filter and BMA above it; this produced a geometric standing waveform pattern on the aspirate fluid surface. The frequency was manually adjusted as the remaining unconcentrated volume reduced to maintain a standing wave and the resultant stable cell suspension. A vacuum pressure (25 psi) was applied from below the filter, to filter out preferentially the smaller cells (erythrocytes, diameter 5–8 μm, and platelets, 1–3 μm), while retaining the NC fraction above the filter. After filtration, the volume of aspirate remaining above the filter was recovered as concentrated BMA for analysis (Figure 1B, iv). Before filtration, 2–5 mL of fresh heparinized BMA was set aside as unconcentrated control BMA.

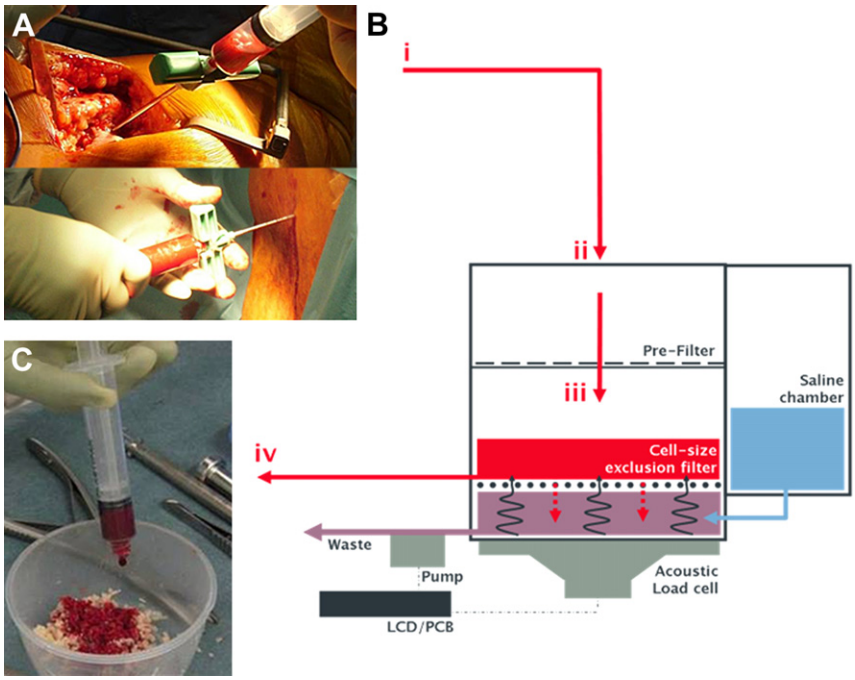

Figure 1. Osteoprogenitor enrichment strategy. (A) BMA from the femoral canal obtained during a total hip replacement (top) and from iliac crest (bottom). (B) BMA enrichment process. Immediately after aspiration and within the sterile field (i), BMA is injected into the unit (ii). After passing through a pre-filter to remove debris, the BMA is applied to a secondary filter (iii), and an acoustic wave is applied to agitate the sample and ensure an even cell suspension. Negative pressure draws erythrocytes and platelets through the filter retaining NC fraction via size exclusion. Enriched NCs, retained above the filter, are removed for therapeutic application (iv). (C) Concentrated bone marrow being applied to allogeneic bone graft (allograft) before bone grafting. LCD, liquid crystal display; PCB, printed circuit board.

Filtration time

Filtration was continued either to achieve a minimum 4.0-fold volume reduction or until flow through the filter became laborious. Start and end time points and starting volume and final volume were recorded for each sample filtered.

NC count and cell viability analysis

NC counts of unconcentrated and concentrated BMA were determined on 18 samples using Guava EasyCyte flow cytometry (Millipore, Watford, UK) and confirmed by manual count on a hemocytometer. Guava ViaCount Reagent (Millipore) was used to differentiate live and dead cells, and the viable cell count value was used in all calculations. To confirm NC enrichment relative to erythrocytes, a single donor (female, 55 years old) was filtered to 50% and 25% starting volume, and NCs were counted relative to erythrocytes using Guava EasyCyte flow cytometry. Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, Bellevue, WA, USA) and SPSS Ver. 18 (IBM United Kingdom Ltd., Hampshire, UK).

CFU-F analysis

CFU-F assay was used as an indicator of osteoprogenitor number present in the aspirate before and after filtration through the device and was performed on 15 samples (Table I). After determination of viable NC count using Guava EasyCyte flow cytometry, unconcentrated and concentrated BMA was seeded into T25 culture flasks ($n = 3$) according to the Friedenstein protocol for density independent growth (25). Briefly, aspirates diluted in basal media (αMEM containing 10% fetal calf serum [FCS]) to achieve clonal seeding densities of 1×10^4, and 1×10^5 NCs/mL were strained to ensure a single cell suspension and seeded at 0.2 mL/cm^2. After 3 h at 37°C and 5% carbon dioxide (CO_2), plates were washed twice with PBS to remove non-adherent cells and incubated at 37°C and 5% CO_2 in basal media for 14 days. After fixation in 95% ethanol and staining for alkaline phosphatase activity against Gill's hematoxylin nuclear counter-stain, colony number was counted, and the CFU-F/mL value for each aspirate was calculated taking into account the dilution factor required to achieve clonal seeding densities. Parallel assays of like-for-like volumes of unconcentrated and concentrated BMA were also performed to illustrate differences in CFU-F concentration.

Flow cytometry analysis of stem cell markers

Unconcentrated and concentrated BMAs from a single donor (male, 59 years old) were washed in

Table I. Nucleated cells and CFU-F concentrations before and after filtration.[a]

				Pre-filtration		Post-filtration	
Sex	Age	Aspirated volume (mL)	Volume fold concentration	Viable NC concentration (million cells/mL)	CFU-F concentration (per mL BMA)	Viable NC concentration (million cells/mL)	CFU-F concentration (per mL BMA)
F	77	20	2.4	6.6	71	13.2	141
M	75	40	4.7	6.1	29	23.8	143
F	91	18	2.7	6.8	89	14.9	198
F	70	40	4.0	27.8	115	89.3	357
M	70	19	5.2	12.5	158	43.7	554
F	82	20	4.0	19.6	116	41.0	611
F	78	20	4.0	16.9	202	78.1	703
F	75	40	5.5	44.8	131	195.8	703
F	71	40	4.0	17.7	215	59.2	977
F	69	20	4.0	14.2	426	52.3	1152
M	55	40	4.0	12.1	317	40.3	1379
F	89	20	6.0	32.4	330	126.0	1418
M	52	20	4.0	18.5	368	61.6	1493
F	78	40	4.3	37.7	2108	95.4	3955
M	68	20	4.0	70.2	1032	234.1	4003
Mean	73.3	27.8	4.2	22.9	380.3	77.9	1185.8
SD	10.5	10.3	0.9	17.4	536.9	64.0	1220.9
Median	73.3	20	4	17.66	214.59	61.63	977.34
IQR	8.5	20	0.49	16.75	225.56	50.01	815.97

F, female; IQR, interquartile range; M, male; SD, standard deviation.
[a]Concentrations corrected for BMA dilution with heparin at time of harvest. Samples below the dotted line displayed post-filtration CFU-F concentrations above the critical therapeutic threshold of 1000 CFU-F/mL suggested by Hernigou *et al.* (23).

PBS with 1% bovine serum albumin (BSA) before being incubated in blocking buffer (PBS, 1% bovine serum albumin, 10% human serum, 5% FCS) at room temperature for 30 min. Cells were immunolabeled with antibodies against Stro-1 (undiluted culture supernatant from the STRO-1 hybridoma originally provided by Beresford, University of Bath), CD146 (1:50, mouse monoclonal [P1H12], BD Biosciences, San Jose, CA, USA) and CD105 (1:50, chicken polyclonal, Abcam, Cambridge, UK) for 60 min at room temperature and then washed and incubated with fluorescently labeled secondary antibodies (1:100). Labeled populations from each sample were quantified against the relevant isotype control using Guava EasyCyte flow cytometry.

Multi-lineage differentiation of concentrated and unconcentrated populations

Following a 3.22-fold NC concentration, equal volumes of unconcentrated and concentrated aspirate from a single donor (female, 70 years old) were seeded across culture vessels and culture expanded for 12 days. After expansion over 14 days, cells (which were at <80% confluence) were trypsinized, counted and reseeded in quadruplicate, at densities consistent with the fold difference in cell number (2.54-fold) obtained from expanded concentrated versus unconcentrated samples, into 12-well plates for osteogenic, adipogenic and chondrogenic differentiation. Control cultures were refreshed with basal media every 3 days.

Osteogenic culture

After a further 14 days in basal conditions, monolayer cultures of concentrated and unconcentrated BMA were transferred to osteogenic media (αMEM with 10% FCS, 100 μM ascorbate [ascorbic acid 2-phosphate] and 10 nM dexamethasone) for 14 days. Media were refreshed every 2–3 days.

Adipogenic culture

After a further 14 days in basal conditions, monolayer cultures of concentrated and unconcentrated BMA were transferred to adipogenic media consisting of basal cell culture media containing additional 2 g/L D-glucose and 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 1% ITS solution (equal to 10 μg/mL insulin; Sigma-Aldrich, St Louis, MO, USA) and 100 μM indomethacin for 3 days followed by 1 day in basal cell culture media containing 1% ITS solution. Both cycles were repeated for 28 days.

Chondrogenic culture

Monolayer expanded cells were seeded at high density (unconcentrated BMA 1.475×10^6 cells/mL; concentrated BMA 3.75×10^6 cells/mL) in 20 μL

volumes at the center of each well and, after 30 min incubation to allow cell adhesion, cultured in chondrogenic media (serum-free αMEM supplemented with 10 ng/mL transforming growth factor-β3, 10 nM dexamethasone, 100 μM ascorbate, and 10 μL/mL 100 × ITS solution) for 21 days. Media were refreshed every 2−3 days.

Histologic staining and quantification of differentiated cultures

Osteogenic differentiation was determined using alkaline phosphatase histochemical staining, demonstrated with Naphthol AS-MX Phosphate and Fast Violet B Salt (both Sigma-Aldrich). Relative alkaline phosphatase staining intensity was quantified using CellProfiler image analysis software (Broad Institute, Cambridge, MA, USA). Briefly, low magnification (×25) brightfield color images were taken in quadruplicate at regular points across each well. Positive staining was identified from background using the CellProfiler UnmixColors module. Staining intensity was normalized by setting the minimum intensity of each image to zero and rescaling stain intensities relative to the maximum intensity across all images using the CellProfiler RescaleIntensity module. Accurate representation of each image was confirmed by eye before the mean intensity of each image was measured using the CellProfiler MeasureIntensity module.

Adipogenic differentiation was identified by adipocyte accumulation of lipid droplets observed under light microscopy using a Zeiss microscope (Carl Zeiss, Cambridge, UK) and after staining for lipid with oil red O histochemistry. Briefly, cells were fixed in Baker's formal calcium, rinsed in 60% isopropanol, and stained using double-filtered oil red O solution. After imaging, oil red O staining was leached from the cells in 100% isopropanol, and absorbance was measured via spectrophotometry.

Chondrogenic differentiation was determined by staining for proteoglycan with alcian blue. After imaging, alcian blue staining was leached using 0.5% Triton X-100, and absorbance was measured via spectrophotometry.

Cell seeding onto decellularized human trabecular bone graft material

Decellularized human trabecular bone allograft was prepared as previously described using sequential hydrogen peroxide and PBS washes (26). BMA from the iliac crest of a single donor (male, 39 years old) was filtered through the device to achieve a fold reduction in volume of 7.4. The relatively high fold volume reduction was achieved to facilitate observation of effects over the high variation characteristically observed in studies of three-dimensional seeding efficiency. A corresponding 7.2-fold increase in the concentration of NCs was confirmed via flow cytometry. Equal volumes of unconcentrated BMA and concentrated BMA were applied to bone graft to assess the effect of concentration of BMA on seeding. Two further preparations were applied to bone as controls: (i) a 7.4-fold reduced volume of concentrated BMA to normalize to unconcentrated BMA by NC number and (ii) a 7.4-fold reduced volume of concentrated BMA rediluted in PBS to normalize to unconcentrated BMA by both volume and NC number. Aspirates were applied so as to immerse approximately 5 mm^3 pieces of decellularized human trabecular bone. After an 18 h incubation under gentle agitation, excess BMA was removed, and samples were washed twice with PBS before being reimmersed in αMEM with 10% FCS. Samples were cultured for a further 48 h before cell quantification and viability analysis.

WST-1 assay for relative quantification of cell seeding

After seeding and a 48-h culture period, the extent of cell seeding and growth on decellularized human trabecular bone matrix was assessed via the colorimetric WST-1 assay for metabolic activity (Cat. No. 11644807001; Roche, West Sussex, UK). Seeded matrices were washed twice with PBS, re-immersed in 9% WST-1 reagent and transferred to an incubator. After 2 h, the absorbance was read at 450 nm on an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA).

Cell viability and imaging

Before fixation, cell cultures were incubated for 45 min (37°C and 5% CO_2) in the presence of αMEM containing 10 μg/mL CellTracker Green and 5 μg/mL Ethidium Homodimer-1 (both Invitrogen, Carlsbad, CA, USA) to label viable and necrotic cells. Cells were incubated in fresh αMEM for a further 45 min before fixing in 95% ethanol for 15 min. Fluorescent imaging was conducted on a Zeiss Axiovert 200 inverted microscope equipped with an AxioCam MRm monochrome camera and an X-Cite 120 fluorescence light source (all Carl Zeiss, Cambridge, UK).

Results

Femoral BMA sampled from 15 patients from elderly cohort was concentrated 4-fold with corresponding enrichment of osteoprogenitors

Fifteen samples were successfully filtered, after which further analysis was performed. Samples were

obtained from 5 male and 10 female patients, with a mean age of 73 years (range, 52–91 years). All samples were analyzed for NC number and viability and cultured for CFU-F analysis (Table I). No significant difference in filtration time, NC count or colony-forming efficiency was observed between the sexes or across the age range of this elderly cohort (data not shown).

The 15 freshly obtained samples of BMA were filtered to achieve a 2-fold to 6-fold reduction in volume (mean = 4.2) with an average filtration time of 18.23 min. Eight samples were filtered to achieve the target 4-fold volume reduction, and five samples were filtered to achieve >4-fold volume reduction. Counts of viable cells after a 2-fold and then 4-fold reduction in volume revealed a corresponding increase in the concentration of NCs in the concentrated aspirate relative to both volume and erythrocyte concentration (Figure 2A). Flow cytometry analysis of commonly used markers for osteoprogenitors revealed enrichment of these cells in the concentrated BMA volume (Figure 2B), and an increase in the concentration of CFU-F was observed via colony-forming efficiency analysis (Figure 2C). A viable NC concentration of mean 3.4 (±0.69) was observed after 4-fold volume reduction (Figure 2D) indicating 82% (±16.93) of NCs were recovered after filtration (Figure 2E). An equivalent CFU-F concentration of 3.9 (±0.83) was observed (Figure 2F). When BMA was filtered to achieve >4-fold volume reduction, a decline in NC recovery (mean = 69.1% ± 10.6) was observed (Figure 2E).

Concentrated BMA displayed increased osteogenesis and chondrogenesis and reduced adipogenesis in vitro

After a 3.22-fold concentration of NCs via acoustic-mediated filtration, concentrated and unconcentrated BMAs from a single patient were seeded at an equal volume and cells were expanded before being transferred to differentiation conditions to assess the effect of cell concentration on the response to multilineage induction. Cells from concentrated BMA seeded onto tissue culture plastic at an equal volume to unconcentrated BMA displayed significantly higher alkaline phosphatase activity ($P < 0.05$) in both osteogenic and basal conditions (Figure 3A). When cells obtained from concentrated versus unconcentrated BMA were seeded at equivalent cell concentrations into chondrogenic high-density culture conditions, significantly

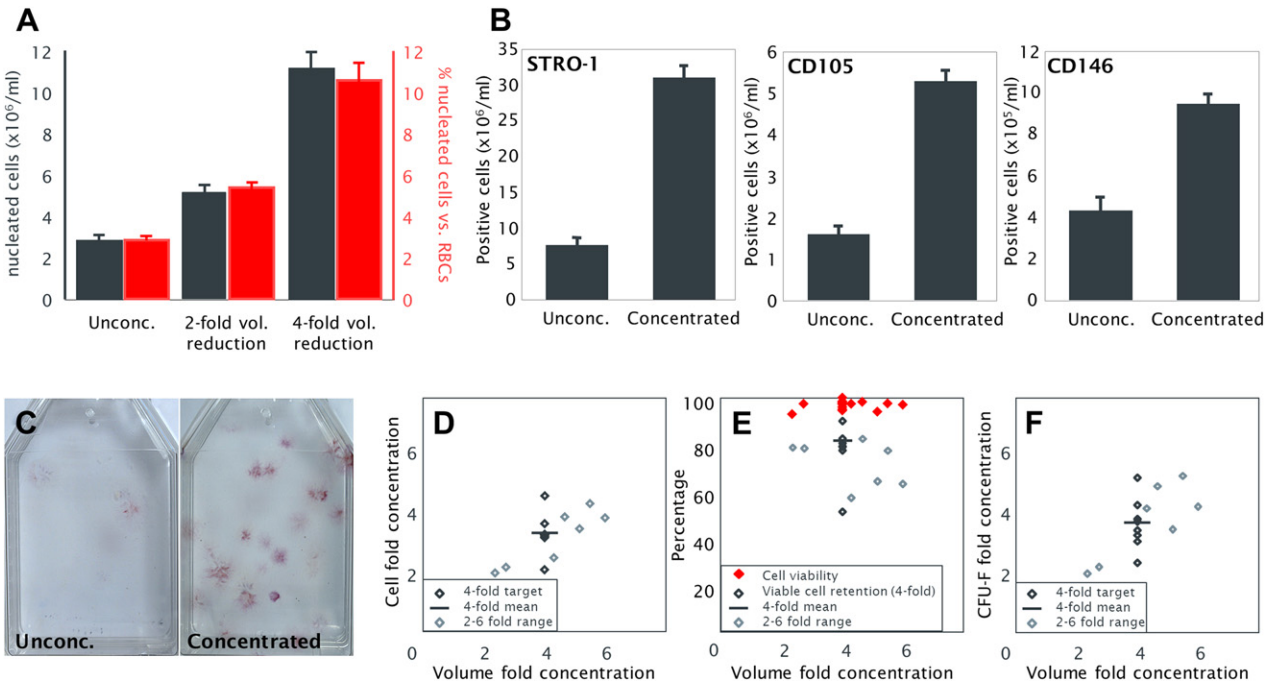

Figure 2. Osteoprogenitor enrichment of BMA. (A) After filtration to achieve 2-fold and 4-fold volume reductions, a corresponding concentration of total NCs relative to erythrocytes/ RBCs was observed. Error bars: ± standard deviation of replicates. (B) Flow cytometry analysis of BMA from a different patient (unconcentrated NC count = 14.5 × 10^6/mL) immunolabeled for markers associated with osteoprogenitors revealed a corresponding enrichment in concentrated samples. Error bars: ± standard deviation of replicates. (C) Equal volumes of unconcentrated and concentrated BMA were cultured for 14 days, and colonies were counted after staining for alkaline phosphatase. In this case, a 3.7-fold increase in CFU-F was observed. (D) Across the 15 samples, a corresponding enrichment of total NCs was observed in relation to the volume filtered. (E) Although cell viability was maintained during filtration, a reduction in the percentage retention of NCs was observed when samples were filtered to achieve >4-fold reduction in volume. (F) A corresponding enrichment of CFU-F relative to the aspirate volume filtered was observed.

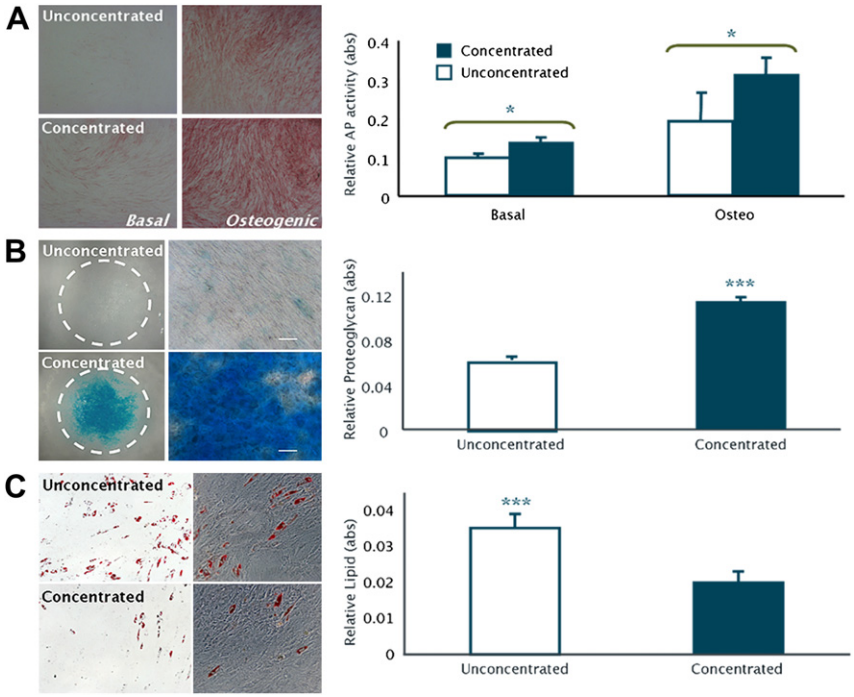

Figure 3. Multilineage induction of human bone marrow stromal cell cultures derived from concentrated versus unconcentrated BMA. Following a 3.22-fold concentration, aspirates were seeded at equal volumes and cells expanded before being transferred to differentiation conditions. Enhanced osteogenesis (A) (alkaline phosphatase activity; $*P < 0.05$) and chondrogenesis (B) (proteoglycan; $***P < 0.001$) but reduced adipogenesis (C) (lipid; $***P < 0.001$) were observed in concentrated samples relative to unconcentrated samples. (abs, absorbance.)

higher proteoglycan synthesis ($P < 0.001$) was observed in concentrated samples with negligible alcian blue staining for proteoglycan being observed in cultures derived from and seeded at densities equivalent to unconcentrated BMA (Figure 3B). In contrast, under adipogenic conditions, higher density seeding facilitated by BMA concentration was observed to reduce significantly ($P < 0.001$) the response to adipogenic induction compared with unconcentrated BMA (Figure 3C).

Osteoprogenitor enrichment of BMA improves cell-seeding efficiency onto bone graft

To assess the effect of concentration of BMA on the seeding efficiency of adherent cells onto allogeneic bone graft, 25 mL of freshly obtained aspirate was filtered to achieve a 7.4-fold reduction in volume. Total NC counts revealed a 7.2-fold concentration of NCs with 97.8% viability corresponding to a 95.1% viable NC retention efficiency. Equal volumes of concentrated ($Conc._{vol.}$) and unconcentrated (Unconc.) BMA were seeded onto decellularized human trabecular bone and, after culture, assessed for relative seeding efficiency via imaging of viable cells and an assay for relative metabolic activity (Figure 4A). To control for the various possible factors affecting seeding efficiency, two further control samples were prepared: concentrated BMA was added at 7.2-fold reduced volume to normalize for cell number and assess for the effect of concentration alone ($Conc._{cell}$), and a 7.2-fold reduced volume of concentrated BMA was re-diluted in PBS to normalize for volume and cell number and assess for the effect of NC concentration relative to erythrocytes alone ($Conc._{cell+vol.}$). In all cases, concentrated BMA demonstrated significantly higher seeding efficiency than the unconcentrated control (Figure 4B,C). $Conc._{vol.}$ resulted in the highest seeding reflecting the increased number of NCs present in these samples; however, concentration resulted in significantly higher seeding even when normalized for cell number ($Conc._{cell}$ and $Conc._{cell+vol.}$). The fact that the increase in seeding efficiency was not lost when concentrated BMA was re-diluted ($Conc._{cell+vol.}$) indicates that the enhanced seeding observed in the samples normalized to the control by cell number can be attributed to concentration of NC number relative to erythrocytes.

Discussion

In the current study we assessed the potential of an acoustic wave-facilitated filtration technique to enrich BMA from an elderly cohort for the NC population, and tested the hypothesis that such cell enrichment enhances the regenerative efficacy of

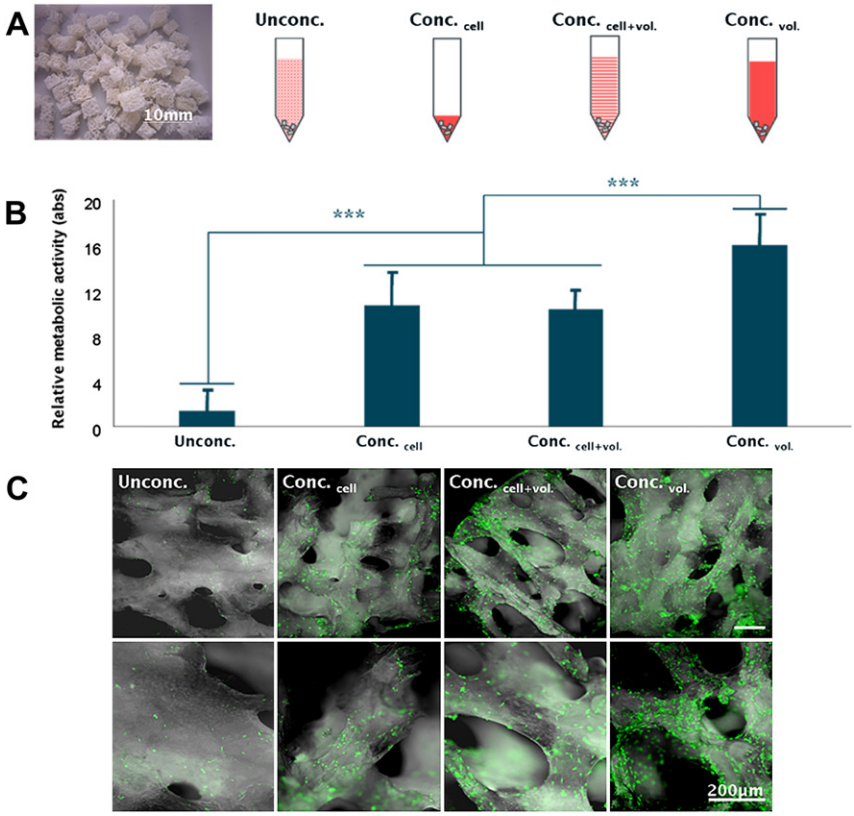

Figure 4. Enhanced cell seeding efficiency via BMA enrichment. BMA was concentrated and seeded, at equal volumes, onto allograft ($Conc._{vol.}$) to compare seeding efficiency relative to unconcentrated controls (Unconc.). In addition, concentrated BMA was normalized to the control by NC number ($Conc._{cell}$) or normalized by cell number and volume by redilution ($Conc._{cell+vol.}$) to assess the effect of concentration apart from cell number and concentration relative to erythrocytes (A). In all cases, seeding efficiency is significantly higher (***$P < 0.001$) when BMA is concentrated, as observed via WST-1 assays for gross metabolic activity (B) and CMFDA-1 staining (C). (Scale bars = 200 µm.) (abs, absorbance.)

BMA. This study demonstrated effective volume reduction in BMA from the femoral canal of a cohort of elderly patients. After filtering the sample to achieve a 4-fold reduction in volume, a corresponding 3.4-fold enrichment of viable NCs and a 3.9-fold enrichment of CFU-F was observed (difference not significant), demonstrating this device to be effective in the intended function of osteoprogenitor enrichment. The average filtration time of 18 minutes proved the viability and practicality for intraoperative clinical application of the device.

There was a wide variation of CFU-F/mL from the aspirated marrow of this cohort of patients (29-2108 CFU-F/mL), although a wide range was also seen in a young cohort previously tested (710−1440 CFU-F/mL) (JN Ridgway, SJ Curran, unpublished observations, 2010). These values are also consistent with a recent study comparing CFU-F numbers obtained from the iliac crest and the femoral canal (27). Dilution of marrow with peripheral blood during sampling is also likely to account for some variability (28,29). This may explain the variable outcomes seen in clinical practice and account for the low CFU-F counts after concentration by centrifugation in the seven cases of non-union in the series by Hernigou *et al.* (23). Initial osteoprogenitor number in BMA from our cohort of elderly patients had a mean of 380 CFU-F/mL significantly lower than the therapeutic threshold of 1000 CFU-F/mL suggested by Hernigou *et al.* (23).

A phenomenon of age-related decline in CFU-F number has been suggested by some authors (16,30), although other studies have demonstrated maintenance of osteoprogenitor number and function with aging, including in osteoarthritic and osteoporotic bone (31−33). Nevertheless, the clinical indication for bone tissue engineering technology is most applicable to elderly patients, and the low CFU-F number found in this cohort of elderly patients highlights the pressing need for cell enrichment strategies in clinical practice. Enrichment of CFU-F by 3.7-fold over all samples filtered increased the mean value in concentrated aspirate to 1185 CFU-F/mL. There was a close correspondence between volume reduction and concentration of viable NC count relative to both total volume and erythrocyte

number indicating good specificity of filtration. Additionally, enriched aspirate contained significantly higher concentrations per milliliter aspirate of cells expressing three widely used osteoprogenitor cell markers.

Following significant previous clinical and pre-clinical studies indicating the importance of osteoprogenitor concentration for bone repair (10,19,20,22,23,34), we designed two *in vitro* experiments to probe the regenerative significance of BMA cell concentration by assessing the effect of cell concentration on skeletal differentiation and seeding efficiency. After culture in osteogenic, chondrogenic and adipogenic differentiation conditions, an enhanced osteogenic and chondrogenic response but reduced adipogenic differentiation was observed in concentrated populations compared with populations derived from unconcentrated BMA. These significant differences, which reflect differences of initial cell-seeding density between treatments, were observed despite a relatively small fold difference in cell number (2.54) after the initial phase of expansion. These results underline the importance of cell concentration for the outcome of skeletal regeneration strategies. The importance of higher cell density for chondrogenic differentiation is well established and provides the rationale for micro-mass culture approaches to chondrogenic differentiation (35–37); however, the influence of initial cell-seeding density on osteogenic and adipogenic differentiation remains controversial (38–42). The reduced adipogenic responsiveness observed in this study may be a function of the increased levels of alkaline phosphatase activity, indicating early osteogenesis, observed in basal cultures of concentrated BMA.

The seeding of cells onto three-dimensional scaffolds and matrices is an important step in many tissue engineering approaches. Several recent studies have made use of allograft or allograft substitute materials seeded with BMA (11,12,20,43), so the demonstration that cells enriched by the acoustic filtration method adhere in greater numbers onto allograft after enrichment is highly significant. In the current study, we have demonstrated a significantly greater enhancement of seeding efficiency in concentrated BMA compared with unconcentrated BMA. We have shown that the effect is not only a function of higher NC number within a given plasma volume, although this is itself an important observation in a technique where volume is often a limiting factor, but also that seeding efficacy is improved critically as a function of reduced erythrocytes. This finding is consistent with a previous study demonstrating the positive effect of erythrocyte lysis on CFU-F growth (28) and indicates that even in a situation where cell number is limiting, filtration of erythrocytes is itself beneficial for tissue engineering strategies using BMA. The negative impact of erythrocyte number on seeding efficiency may offer an insight into why concentration of osteoprogenitors is of regenerative significance independent of total number of osteoprogenitors delivered.

The current study provides evidence for the importance of BMA NC concentration for cell differentiation and seeding of scaffolds using an approach designed to be applicable intraoperatively. Substantial heterogeneity remains within this fraction with the CFU-F population constituting, in this cohort, a mean of only 0.012% (±0.013%) of total NCs. Furthermore, well-documented functional heterogeneity exists within the CFU-F fraction itself (44,45). There is thus considerable scope for further enrichment of osteoprogenitors, and a large body of research is devoted to developing approaches to achieving this. It should be noted however, that while this and previous studies (10,20,22,23,28) have indicated the importance of CFU-F concentration for regenerative outcome, the increments of benefit derived from further enrichment steps are yet to be defined experimentally in the current context of bone grafting, and the relationship of concentration and efficacy is not necessarily straightforward (46,47). Further work is therefore required to confirm the benefits of further enrichment in balance with the inherent risks of increasingly involved processes.

In conclusion, given the demographics of an increasing and aging population, there is a pressing need for the translation of efficient reconstructive strategies suitable for therapeutic use. Current successful intraoperative strategies for administration of concentrated BMA exploit the available time interval between initial marrow aspiration at the start of the operation and implantation toward the end of the procedure; this allows approximately 20–30 minutes to prepare the aspirate. Gradient centrifugation approaches allow preparation of BMA within this time frame and are the current choice for intraoperative concentration, with several successful systems in routine use (MarrowStim [Biomet, Warsaw, IN, USA)], Res-Q [ThermoGenesis, Rancho Cordova, CA, USA], SmartPReP 2 [Harvest Technologies, Plymouth, MA, USA], Biosafe [Sepax Technologies, Newark, DE, USA]). These techniques have shown proven benefits *in vitro* (48,49), in animal models (50,51) and in a variety of different clinical indications including osseous defects, osteonecrosis, osteoarthritis and peripheral vascular disease (11,20,23,43,52,53). However, despite the success of this technique, centrifugation approaches entail considerable capital equipment expenditure and, due to their size and lack of sterility, preclude BMA concentration within the surgical field risking infection as well as donor cross-contamination

(24,28). We have presented an efficient and inexpensive solution to cell enrichment amenable for development toward a single-use device and able to be applied intraoperatively within the sterile field, offering considerable potential for clinical benefit.

Acknowledgments

This work was funded by the Technology Strategy Board and Engineering, and Physical Sciences Research Council (TS/G001650/1). We would like to thank the orthopedic surgeons from University Hospital Southampton NHS Foundation Trust for provision of bone marrow aspirates.

Disclosure of interest: JID, JOS, AA, DGD and ROC have no conflicts of interest. JNR is employed by Smith & Nephew Ltd., who have a financial interest in the cell concentration method. SJC was previously employed by Smith & Nephew during the initial phase of this project.

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