■ TRAUMA: RESEARCH

The use of the reamer-irrigator-aspirator to harvest mesenchymal stem cells

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The scarcity of mesenchymal stem cells (MSCs) in iliac crest bone marrow aspirate (ICBMA), and the expense and time in culturing cells, has led to the search for alternative harvest sites. The reamer-irrigation-aspirator (RIA) provides continuous irrigation and suction during reaming of long bones. The aspirated contents pass via a filter, trapping bony fragments, before moving into a 'waste' bag from which MSCs have been previously isolated. We examined the liquid and solid phases, performed a novel digestion of the solid phase, and made a comparative assessment in terms of number, phenotype and differentiation capacity with matched ICBMA.

The solid fraction from the filtrate was digested for 60 minutes at 37°C with collagenase. Enumeration was performed via the colony-forming unit fibroblast (CFU-F) assay. Passage (P2) cells were differentiated towards osteogenic, adipogenic and chondrogenic lineages, and their phenotypes assessed using flow cytometry (CD33, CD34, CD45, CD73, CD90, and CD105).

MSCs from the RIA phases were able to differentiate at least as well as those from ICBMA, and all fractions had phenotypes consistent with other established sources. The median number of colonies for the three groups was: ICBMA = 8.5 (2 to 86), RIA-liquid = 19.5 (4 to 90), RIA-solid = 109 (67 to 200) per 200 µl. The mean total yield of cells for the three groups was: ICBMA = 920 (0 to 4275), RIA-liquid = 114 983 (16 500 to 477 750), RIA-solid = 12 785 (7210 to 28 475).

The RIA filtrate contains large numbers of MSCs that could potentially be extracted without enzymatic digestion and used for bone repair without prior cell expansion.

Mesenchymal stem cells (MSCs) are highly proliferative stromal cells that can undergo differentiation into various lineages, including bone and cartilage tissue. They hold considerable therapeutic promise for the development of regenerative medicine.¹⁻⁴ In relation to orthopaedics, MSCs are associated with accelerated bone healing in the experimental setting. They may also have a role in treating nonunions⁵ and enhancing the healing rates of fresh fractures.⁶

This potential has been tempered by the scarcity of MSCs in iliac crest bone marrow aspirate (ICBMA),² from which cells were historically obtained, and the requirement for significant numbers of MSCs for tissue engineering and healing of nonunions.^{5,7} Although MSCs can be expanded significantly by *in vitro* culture with relative ease, with a 100-fold increase in numbers within two to three weeks,⁸ this results in daughter cells that have reduced differentiation capacity⁹ and impaired cell function, with associated growth arrest and a propensity to undergo apopto-

sis.¹⁰ From the practical side this also entails two-stage procedures for orthopaedic interventions, which is both costly and time-consuming.

This has prompted a search for alternative, higher-yield sources of MSCs, where minimal or no cellular expansion would be required to attain sufficient cell numbers. The first step towards such a procedure was the development of concentration devices where a large volume of iliac crest marrow aspirate can be centrifuged to reduce its volume but increase yields of MSCs.¹¹ An alternative method of increasing yields of MSCs is collagenase digestion of solid connective tissue, which appears to release large numbers of MSCs from adipose tissue^{12,13} and trabecular bone.^{3,14,15} However, the use of bacterially derived collagenase, and the comparatively lengthy procedure that digestion entails, has hampered the approval of such methods.

An interesting development that might circumvent the use of collagenase has recently been described employing a new harvesting

Fig. 1

The Reamer-Irrigator-Aspirator.

technique for MSCs using a novel reaming system, the Reamer-Irrigator-Aspirator (RIA) (Synthes, Westchester, Pennsylvania), with MSCs being isolated from the discarded filtrate (Fig. 1).¹⁶ In that report the numbers of MSCs 'were not estimated' and no comparison was made, with those from ICBMA. Additionally, the reaming was performed in an experimental manner, with a reamer of small diameter (12 mm), in elderly patients with wide intramedullary canals who were undergoing hemiarthroplasty of the hip.

The RIA has been used at our institution since 2007 to reduce the incidence of fat embolism and thermal necrosis,¹⁷⁻¹⁹ and as a harvester of autogenous bone graft for the treatment of nonunions.²⁰ Our clinical impression is that fatty material, presumably owing to its deformable nature, is also collected in the discarded filtrate bag (Fig. 1).

The aim of this study was to undertake comparative enumerative, functional and phenotypical studies of MSCs in the liquid component of the RIA filtrate in relation to matched ICBMA. In particular, the solid fatty material found in the filtrate was enzymatically digested and the liberated MSCs were characterised in relation to both the

ICBMA and RIA fluid fraction cells, the main aim of the work being to determine whether large numbers of MSCs could be easily obtained for fracture repair.

Materials and Methods

Approval from the local ethics committee was obtained and all patients provided informed consent. Six patients (one woman) with a mean age of 47 years (28 to 61), five of whom had nonunion of the tibia and one of the fibula, underwent collection of bone graft from the femoral endocortex using the RIA. Technical guidance on the use of the RIA has been previously published.¹⁹ Briefly, the pre-operative radiographs were assessed, a percutaneous approach was made to the greater trochanter and a 'single-pass' technique, with continuous intra-operative imaging, used to ream the canal of the femur and endocortex, aiming to remove 1.5 mm at the isthmus. Constant irrigation was provided with sterile saline and the intramedullary contents were aspirated via the open-pore filter (Biomet Redi-flow; Biomet, Warsaw, Indiana) into the filtrate bag. Additionally, patients had a matched 10 ml of ICBMA removed from the anterior iliac crest in a single aspiration, using an

Table I. General properties of the different fractions (n = 6)

	Iliac crest bone marrow aspirate		RIA liquid		RIA solid	
	Mean	Range	Mean	Range	Mean	Range
Volume (ml)	10 (0)	NA*	754	190 to 1300	24.3	10 to 85
WCC† (10 ⁶ cells/ml)	32 (10)	18 to 43	8.8	1.4 to 31.4	45	18 to 83.2

* NA, not applicable

† WCC, white cell count

11-gauge, bevel-tipped trocar (Stryker, Kalamazoo, Michigan) and a 10 ml syringe (BD Biosciences, San Jose, California). The ICBMA was transferred immediately into EDTA-containing vacutainers (BD Biosciences).

Samples were processed under aseptic conditions with the RIA filtrate being divided into solid and liquid phases by passage through a 70 µm cell strainer (BD Biosciences). The resulting solid phase was digested using collagenase (Stem Cell Technologies, Vancouver, British Columbia) at a 1:1 ratio w/v (final concentration = 0.125%) (60 min at 37°C). The three different fractions, namely ICBMA, RIA liquid and RIA solid, from each donor, were then passed through a 70 µm cell strainer to remove clots, undigested fat and bone fragments, and following red-cell lysis in 0.4% acetic acid (Sigma), a manual white cell count was performed.

Samples were plated without prior mononuclear cell (MNC) concentration as previously described²¹ for colony-forming unit fibroblast (CFU-F) assay and cellular expansion. This removes a step that has the potential for variability and loss of MSCs, and was performed in duplicate on a volumetric basis, with 200 µl of each fraction (400 µl of the RIA solid fraction to account for dilution with collagenase) plated directly into a 10 cm diameter Petri-dish (Corning, Acton, Massachusetts) with 15 ml of non-haematopoietic media (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were allowed 48 hours to become adherent, after which red cells were removed with two phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, California) washes. Adherent cells were allowed to grow at 37°C with 5% CO₂, with twice-weekly half-media changes. The CFU-F assay was performed on the 14th day, and parallel cultures underwent further expansion. This assay is a standard technique and was performed as previously described.²²

Cellular expansion was performed to produce sufficient cells to undertake trilineage differentiation in all fractions using previously described techniques.²² For this differentiation, passage-2 MSCs (four donors) were induced towards osteogenesis, chondrogenesis and adipogenesis according to standard protocols.²² Osteogenesis was assessed at 14 days, testing for alkaline phosphatase activity as previously described,²² with the total calcium assessed in parallel cultures at 21 days. The use of these *in vitro* assays as an indirect measure of osteogenesis is well accepted and frequently reported.^{2,22}

Cultures were digested in 0.5 M HCL (Sigma Aldrich, St Louis, Missouri) for four hours at 4°C, and calcium concentrations were measured quantitatively using a Sentinel Calcium kit (Sentinel Diagnostics, Milan, Italy). Adipogenesis was assessed at 21 days using oil-red-O staining as previously described.²² Chondrogenesis was assessed at 21 days, measuring GAG/DNA, using a Quant-iT PicoGreen dsDNA Reagent Kit (Molecular Probes, Invitrogen), and glycosaminoglycan (GAG) was measured using a Blyscan kit (Biocolor Life Sciences, Belfast, United Kingdom) as previously described.²³

For surface phenotyping of culture-expanded MSCs (P2), passage-2 MSCs were trypsinised using standard techniques²² and the cells suspended at 10⁷ cells per ml in fluorescence-activated cell sorting buffer (PBS +0.5% BSA). The cells were fluorescently labelled with the following antibodies: CD33-FITC, CD34-PerCp, CD73-PE, CD45-PE-Cy7, CD19-PE and CD61-FITC (all from BD Biosciences), CD271-APC (Miltenyi Biotec), CD31-FITC, CD105-PE and CD90-PE (all from Serotec), at the manufacturer's recommended concentrations, and incubated for 15 minutes in the dark at room temperature. Subsequently, the unbound antibodies were removed by washing in FACS buffer and the cells analysed on the LSRII flow cytometer (BD Biosciences).

Statistics. Statistical analysis and graphing were performed using GraphPad Prism version 4.00 for Windows (GraphPad, La Jolla, California). A Gaussian distribution could not be assumed, given the number of samples, and the differences between the pooled results were investigated using the Kruskal-Wallis test, with differences between groups tested with Dunn's multiple comparison test. Significance was assumed when $p < 0.05$. Spearman's correlation coefficient was calculated to investigate the relationship between data sets, with $r > 0.6$ considered significant.

Results

The general properties of the different fractions are summarised in Table I, which shows large variations in volume and cellular content within the RIA liquid and solid fractions, as demonstrated by the SD. This variation may be partially explained by patient heterogeneity, varying degrees of reaming, and dilution with saline. The mean volumes of RIA liquid far exceeded those of ICBMA and RIA solid fractions (approximately 70 and 30 times, respectively), although this fraction possessed a corresponding

Fig. 2a

Fig. 2b

Fig. 2c

The CFU-F assay. Figure 2a – microscopy at seven days showing growing MSCs with long projections and prominent nucleoli in all fractions (arrows), surrounded by smaller round haematopoietic cells. Figure 2b – macroscopic examples of CFU-F staining. Figure 2c – CFU-F per 200 µl (horizontal lines mark median values). Of note: all of the samples are matched across different sizes in each case.

Table II. Yields of colony-forming unit fibroblast (CFU-F)

Sample	CFU-F per ml		CFU-F total yield	
	Iliac crest bone marrow aspirate	RIA liquid	RIA solid	
A	15	21 000	7210	
B	105	38 950	10 000	
C	45	16 500	8625	
D	40	83 700	28 475	
E	10	52 000	9400	
F	430	477 750	13 000	
Mean	92	114 983	12 785	
SD	153	179 364	7922	
Median	40	45 475	9700	

lower white cell count of approximately one-quarter of the other fractions.

Enumeration/CFU-F assay. Plastic adherent cells, which had morphologies that were consistent with previous reported sources of MSCs that could be expanded in cell culture, were found in all three fractions² (Fig. 2). The CFU-F assay showed that the RIA liquid and ICBMA fractions had comparable numbers of colonies per 200 µl of sample, at 36 and 38, respectively; $p = 0.1$. However, there were more MSCs per 200 µl within the RIA solid fraction than in the other fractions: this reached significance when compared to ICBMA (36 *vs* 136 per 200 µl, $p = 0.03$) (Fig. 2). We postulated that the number of MSCs within the RIA liquid fraction might be related to the volume of liquid collected with fewer MSCs per 200 µl with greater amounts of dilution. However, when this relationship was examined by linear regression analysis, no correlation was found.

The total yield of uncultured ‘fresh’ MSCs for fracture repair is important. Although a ‘total yield’ figure is appropriate for RIA-derived sources, a yield per 10 ml is correct for the ICBMA sample (Table II). This showed nearly five times higher numbers of MSCs in the RIA liquid than in the RIA solid fraction, because of the much higher volumes of the former. The volume of ICBMA required to produce an equivalent number of CFU-Fs to the RIA liquid fraction is 1137 ml, emphasising the large number of MSCs within this fraction.

The CFU-F assay showed that the RIA liquid and ICBMA fractions had comparable numbers of colonies per 200 µl of sample, median values of 8.5 and 19.5, respectively ($p > 0.05$). However, there were more MSCs per 200 µl within the RIA solid fraction than in the other fractions: this reached significance when compared to ICBMA (8.5 *vs* 109 per 200 µl, $p < 0.01$).

Trilineage differentiation. We compared osteogenesis qualitatively between the different fractions using alkaline phosphatase staining (Fig. 3), and quantitatively by measuring the total calcium production (Fig. 3). Alkaline phosphatase staining was positive in all instances, appearing more pronounced within the RIA solid fraction. Similar trends were seen in the measurement of total calcium, although statistical significance was not reached. Although calcium was detected in all RIA liquid and solid samples, it was not found in MSCs from two of the donors’ ICBMAs. Adipogenesis occurred in all instances, with oil-red-O staining lipid vesicles red (Fig. 4). This appeared qualitatively better in the RIA solid fraction and may be related to its presumed adipogenic heritage. Chondrogenesis showed no significant

Fig. 3a

Fig. 3b

Osteogenesis. Figure 3a – photographs showing macroscopic ACP staining. Figure 3b – bar chart showing calcium production per well. Error bars show SD.

differences between the fractions but a trend towards higher levels in the RIA solid fraction (Fig. 4).

Population doubling showed no significant variability between fractions (Table III). This is of note, as differences in trilineage differentiation cannot be attributed to it. Mesenchymal stem cells from all fractions showed similar rates of proliferation, suggesting that these cells, albeit from different niches and techniques of harvesting, had similar properties of propagation (Table III).

The high levels of variability, as illustrated by large error bars, may be related to inter-individual physiological differences. We postulated that the overall trends towards improved differentiation in the RIA solid fraction may have been related to a reduced number of population doublings, secondary to different CFU-F plating densities. However, when we exam-

ined this we found no significant differences in this or in the rate of MSC growth between the different fractions.

Surface phenotype of culture-expanded MSCs (P2). Flow cytometry showed essentially homogeneous populations in all fractions, which were universally negative for CD19, CD31, CD33, CD34, CD45 and CD61, and positive for CD73, CD90 and CD105. These data are consistent with previous reports of MSC cultured phenotypes²⁴ and show that MSCs from these different ‘bone’ niches had similar phenotypes.

Discussion

We examined MSCs from the RIA filtrate and compared them with those obtained from ICBMA in terms of cell frequency, differentiation capacity and cultured phenotype.

Fig. 4a

Fig. 4b

Adipogenesis with oil red-O staining (a) and chondrogenesis showing GAG/DNA (b). Error bars show SD.

Table III. Population doublings (A) achieved at P2 and rates of proliferation in days (B)

	Iliac crest bone marrow	RIA liquid	RIA solid	Mean	SD
A					
A	19	16	14	16	1.99
B	16	18	14	16	1.33
C	16	17	15	16	1.29
D	15	13	16	15	1.25
Mean	17	16	15	16	0.61
SD	1.58	1.94	1.05		
B					
A	1.54	1.84	2.07	1.84	0.22
B	1.70	1.58	1.96	1.78	0.15
C	2.10	1.98	2.23	2.04	0.16
D	2.22	2.58	2.08	2.28	0.20
Mean	1.89	1.99	2.09	1.98	0.07
SD	0.32	0.42	0.11		

We also investigated the effects of collagenase digestion on the previously unidentified solid components found in the filtrate. We isolated MSCs within all the fractions based on their plastic adherence, morphology, surface phenotype, proliferative ability and trilineage potentiality, and confirm that the RIA filtrate is a source of MSCs.¹⁶

The isolation of large numbers of MSCs that require no or only minimal expansion could have important scientific and economic benefits for their use in tissue engineering⁶ and the repair of fracture nonunions⁵ with biologically active cells. This study shows that MSCs isolated either from the liquid or the solid fractions of the RIA filtrate are not inferior to those obtained from ICBMA in terms of their differentiation capacity, and are significantly more numerous.

The large numbers of MSCs in the RIA liquid fraction, which can be released without collagenase digestion, suggest that they may be suitable for direct application in a single-stage procedure, without prior cell culture. The mean number of uncultured MSCs within this fraction exceeds the 50 000 found to provide effective bone repair in one study,⁵ with MSC numbers in the more deficient samples approaching this figure when combined with the RIA solid harvest. Our study suggests that approximately 1 l of ICBMA would be required to achieve similar numbers of MSCs that are in RIA liquid fraction alone, which is not feasible in a clinical situation. The use of a device to concentrate MSCs within ICBMA has also been well described,¹¹ and it is theoretically possible to use a similar device to concentrate the large volume of RIA liquid to a

technically usable amount. Uncultured MSCs can be enumerated via flow cytometry using the marker CD271,²⁵ with magnetic beads processing this marker possibly providing a means of further purifying this source.²⁶

Collagenase digestion of the fatty RIA solid fraction further increases the yield of MSCs that can be achieved from the RIA filtrate. Previous studies have shown that collagenase digestion can release large numbers of MSCs,¹² some reporting 500-fold differences per gram of tissue between ICBMA and lipoaspirate.²⁷ Our results showed an approximate tenfold increase in CFU-F per gram of tissue compared to ICBMA, with this difference possibly being related to the anatomical source and varying methodologies. From a practical perspective, the use of collagenase will prolong the procedure, owing to the delay that tissue digestion entails. Mesenchymal stem cells from all fractions could differentiate towards osteogenic, adipogenic and chondrogenic lineages. Osteogenesis was robust within both RIA fractions compared to ICBMA. The overall low levels of calcium produced, particularly in the ICBMA fraction, may be related to the morbidity of nonunion, and it has previously been suggested that MSCs from patients with nonunion may have inferior MSCs.²⁸ There was a trend towards increased osteogenesis within the RIA solid fraction, which is presumed to originate from fatty bone marrow. This is of interest, as other adipogenic sources have generally been shown to be inferior to ICBMA in forming bone,²⁹ and may be related to an intraosseous location and a potential role in fracture healing. Porter et al¹⁶ described 'robust' chondrogenesis from MSCs from the RIA liquid fractions, although this was not quantified. Our results suggest that chondrogenesis, although present, was no better than from ICBMA. However, we noted a trend towards improved chondrogenesis within the fatty RIA solid fraction, and tentatively suggest that this may relate to MSCs from this fraction having a low-oxygen environment, similar to chondrocytes.

The RIA is frequently used to harvest bone graft in order to obtain osteogenic cells and material for the treatment of nonunions.²⁰ We have shown the osteogenic properties of MSCs derived from the RIA filtrate and have suggested a strategy for concentrating and purifying these cells. The addition of these cells to the harvested bone graft may further improve the concentration of osteogenic MSCs and the chances of union. The use of the RIA for procedures other than the harvest of bone graft, e.g. the prevention of fat embolism, offers an opportunity to harvest this significant source of MSCs for allogeneic or research uses.

The isolation of large numbers of MSCs without collagenase digestion of solid connective tissues provides a situation where these cells have other significant autogenous/allogeneous potential in the repair of connective tissue defects and other MSC-related therapies, such as their auto-immune modifying effect.³⁰ The MSCs released by collagenase digestion from the fatty solid fraction within the RIA filtrate show significant potential with regard to their properties of differentiation and need further study.

MSCs can be found in the RIA filtrate, which is normally discarded as 'waste'. They compare well with MSCs from matched ICBMA in terms of their capacity for differentiation, and are significantly more numerous. The yield of MSCs could be further increased by collagenase digestion of the fatty solid fraction, with cells released in this manner possibly having improved chondrogenic and osteogenic properties. The use of these cells, following concentration or purification, in combination with bone autograft, should be investigated with a clinical trial in the treatment of non-unions. Additionally, cells isolated in this manner should be further examined to see whether they are appropriate for other purported uses of MSCs.

Further opinion

A further opinion by Professor A. Nather is available with the electronic version of this article on our website at www.jbjs.org.uk/education/further-opinions

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