■ TRAUMA: RESEARCH

The viability of cells obtained using the Reamer–Irrigator–Aspirator system and in bone graft from the iliac crest

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We hypothesised that cells obtained via a Reamer–Irrigator–Aspirator (RIA) system retain substantial osteogenic potential and are at least equivalent to graft harvested from the iliac crest. Graft was harvested using the RIA in 25 patients (mean age 37.6 years (18 to 68)) and from the iliac crest in 21 patients (mean age 44.6 years (24 to 78)), after which ≥ 1 g of bony particulate graft material was processed from each. Initial cell viability was assessed using Trypan blue exclusion, and initial fluorescence-activated cell sorting (FACS) analysis for cell lineage was performed. After culturing the cells, repeat FACS analysis for cell lineage was performed and enzyme-linked immunosorbent assay (ELISA) for osteocalcin, and Alizarin red staining to determine osteogenic potential. Cells obtained via RIA or from the iliac crest were viable and matured into mesenchymal stem cells, as shown by staining for the specific mesenchymal antigens CD90 and CD105. For samples from both RIA and the iliac crest there was a statistically significant increase in bone production (both $p < 0.001$), as demonstrated by osteocalcin production after induction.

Medullary autograft cells harvested using RIA are viable and osteogenic. Cell viability and osteogenic potential were similar between bone grafts obtained from both the RIA system and the iliac crest.

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Intramedullary nailing is the standard treatment for diaphyseal long bone fractures of the lower limb. Some authors have suggested that the deposition of autogenous bone graft at the fracture site during reaming increases the rate of union.[1-4] However, whether cells from the reamings remain viable is controversial, as high temperatures[5] and pressures and mechanical damage from reaming may affect the endosteal cells.[6]

The Reamer–Irrigator–Aspirator (RIA; Synthes, Paoli, Pennsylvania) system was initially developed to reduce pulmonary complications associated with reamed femoral intramedullary nailing. The RIA device irrigates the reamer head in order to keep the intramedullary contents cool during vacuum evacuation of the reamed contents.[7] Husebye et al[8] demonstrated lower pressures within the medullary canal when using RIA compared with standard reamers. Since this device can capture reamings with the addition of a filter, it has been used for harvesting intramedullary autogenous bone graft.[2,9-11]

Using a filter system, RIA provides a method of harvesting bone graft that is distinct from iliac crest graft. The disadvantages of iliac crest graft include a limited quantity of graft and donor site morbidity.[12,13] The intramedullary canal of long bones is known to contain many pluripotent cell types,[14] and the osteogenic viability of these cells collected from standard reamers is known.[1,2] However, RIA also adds potential mechanical damage to these cells during irrigation and aspiration. The effluent collected using RIA also contains many osteogenic growth factors,[4,15,16] and the filter system can provide large volumes of bone graft.[17]

Although RIA has been used clinically,[18] there is no evidence to suggest that these cells remain viable. Therefore the questions raised are, is the apparent clinical success related to the growth factors or due to the osteogenic cells?; and how do the cells from the RIA compare with autograft harvested from the iliac crest?

The purpose of this study was to quantify the viability of cells in autograft obtained via RIA and the iliac crest and compare their osteogenic potential. We hypothesised that cells obtained via RIA retain substantial osteogenic potential and are equivalent to cells harvested from the iliac crest.

Patients and Methods

The study received institutional review board approval. Patients were included if they were aged > 18 years and were undergoing a bone

graft harvest procedure, either via the RIA system or from the iliac crest. Exclusion criteria included history of leukaemia, cancer with bone metastases, renal failure, patients on dialysis or receiving immunosuppressive medication, and those with suspected local or systemic infection.

The RIA graft was obtained using a standard technique, which has been previously described.[7] The bony particulate matter retained by the RIA suction filter was used for analysis and the aspirated effluent was discarded. Iliac crest graft was harvested using standard techniques and based on the preference of the surgeon. Standard harvesting techniques included use of curettes and/or acetabular reamers to obtain graft from the iliac crest and wing.[19] At least 1 g of the graft material was placed into saline in a sterile container and transported to the laboratory, where it was processed within two hours.

Cell culture technique. The sample was washed three times with a 2% antibiotic–antimycotic solution (ref. 15240; Invitrogen, Carlsbad, California) in Dulbecco's phosphate buffered saline (D-PBS) and was divided into 0.75 g to 1.0 g samples, leaving behind 0.5 g of sample for primary flow cytometry analysis and cell viability. Some subjects provided ample graft material and were divided into multiple samples, which accounted for the mismatch in number of samples per graft type and total number of patients.

Under aseptic conditions, in a 25 cm^2 flask, 0.75 to 1.0 g of the sample was placed in 8 ml of warm outgrowth medium comprising 10% HyClone Characterised fetal bovine serum (ref. SH3007; Thermo Scientific, Waltham, Massachusetts), 1% antibiotic–antimycotic (ref. 15240; Invitrogen), and 0.05 mg/ml gentamicin in low-glucose Dulbecco's modified Eagle's medium (ref. 11885; Invitrogen).[2] The samples were incubated at 37°C for 28 days. The medium was changed on days four, seven, 11, 14, 18, 21, 25 and 28. On day seven the contents of the flask were emptied, including the bone particles, leaving behind only viable cells. Starting on day 14, the cells were induced for differentiation. Wenisch et al[1] showed that osteogenic differentiation could be achieved with the addition of L-ascorbic acid-2-phosphate and dexamethasone. This differentiation medium was composed of the same ingredients as the outgrowth medium, with the addition of 0.1 µM dexamethasone (ref. D9184; Sigma, St. Louis, Missouri) and 0.05 µM L-ascorbic acid-2-phosphate (ref. A8960; Sigma). On days 11 and 21, 1 ml supernatants per sample were obtained for analysis via enzyme-linked immunosorbent assay (ELISA) to measure the concentration of osteocalcin. These dates were chosen from our pilot data, which indicated that a significant increase between pre- and post-inductive concentrations of osteocalcin occurred between days 11 and 21.

Fluorescence-activated cell sorting (FACS) and cell viability. Fluorescence-activated cell sorting (FACS) was performed on days 0 and 28 to assess the growth and differentiation of the cells. Cell viability was assessed via Trypan blue exclusion on day 0.[20]

The cells at the beginning of the study were taken from a small sample of graft that was withheld from the culture flasks. The cells for FACS analysis at the end of the study were obtained by lifting the confluent layer of cells from the 25 cm^2 flasks at the end of the 28-day culture. The cells were lifted with exposure to 5 ml of warmed AccuTase (Innovative Cell Tech, San Diego, California) for approximately ten minutes. A cell rake was used to assist with removing the adherent cells. These cells were washed with 5 ml of warmed AccuMax (Innovative Cell Tech) to prevent aggregation in preparation for flow cytometry and cell viability evaluation. After lifting the cells, the processing of the initial and final samples was identical.

The graft tissue was placed in a tube with 10 ml of D-PBS. This tube was centrifuged at 1700 rpm for ten minutes at 22°C. The fluid from the tube was aspirated and the cells at the bottom of the tube were isolated using a glass pipette and vacuum suction; 1 ml of D-PBS was then added. At this time 40 µl of this solution were separated into a 2 ml vial containing 40 µl of Trypan blue. From this total of 80 µl, 40 µl were placed on a microscope slide for measurement of cell viability. The cells with uptake of Trypan blue represent cells with disrupted cell membranes and apoptosis (non-viable). Total cells and nonviable cells were measured. Cell viability was calculated by subtracting the number cells that had uptake of Trypan blue (non-viable) from the total number of cells. From this, the volume needed for one million cells was also calculated for FACS analysis. The microlitre of specimen was separated into five test tubes. The first tube was a control: mouse anti-IgG antibody; the four remaining tubes contained antibodies specific to CD34, CD45, CD90 and CD105. These cell surface markers were chosen because mesenchymal stem cells (MSCs) have been shown to express CD90 and CD105, and not CD34 and CD45.[21] The tubes were then incubated with the antibodies for 15 minutes at 10°C. Samples were washed briefly with 500 µl of D-PBS, the tubes were centrifuged for 20 minutes and the fluid was aspirated, leaving behind the cell supernatant which was discarded. At this point, 200 µl of BD Cytofix/Cytoperm (BD Biosciences, Franklin Lakes, New Jersey) was added to the cell pellets. Cytofix/Cytoperm was washed with D-PBS after 15 minutes of incubation at 10°C. Cells were incubated at 10°C for 15 minutes, washed with D-PBS, and centrifuged in preparation for FACS analysis. The tubes were centrifuged a final time prior to FACS analysis. Cell populations were considered to be positive for particular cell surface markers if the fluorescence of that antibody was > 70%; < 15% was defined as negative for cell surface markers. Mouse IgG controls were implemented to account for non-specific antibody adherence.

ELISA for osteocalcin. ELISA for osteocalcin was performed on 1 ml of medium supernatant obtained from the sample flask before the changes of medium on day 11 and day 21, using prepared kits (Quidel Co., San Diego, California). All samples were run in triplicate and the mean calculated.

Table I. Demographics and comorbid factors (RIA, Reamer–Irrigator–Aspirator)

Characteristic	RIA	Iliac crest	p-value
Subjects (n)	25	21	
Mean age (yrs) (range)	37.6 (18 to 68)	44.6 (24 to 78)	0.12
Gender			
Male (n)	18	8	0.0209
Female (n)	7	13	0.0209
Tobacco abuse (n, %)	11 (*44*)	12 (*57*)	0.3745
Alcohol abuse (n, %)	2 (*8*)	2 (*9*)	0.8559
Diabetes (n, %)	1 (*4*)	2 (*9*)	0.4286

Table II. Flow cytometry cell surface marker analysis before and after culture (RIA, Reamer–Irrigator–Aspirator)

	CD34		CD45		CD90		CD105	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
RIA	+	-	+	-	-	+	-	+
Iliac crest	+	-	+	-	-	+	-	+

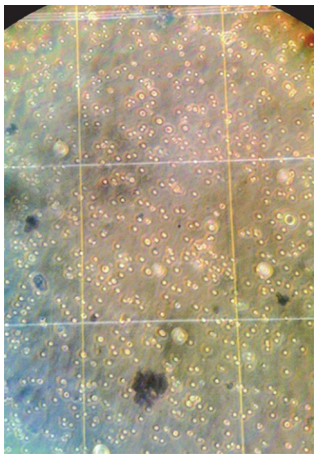

Fig. 1

Cell viability shown through the use of Trypan blue staining. Incorporation of the blue stain in cells indicates apoptosis.

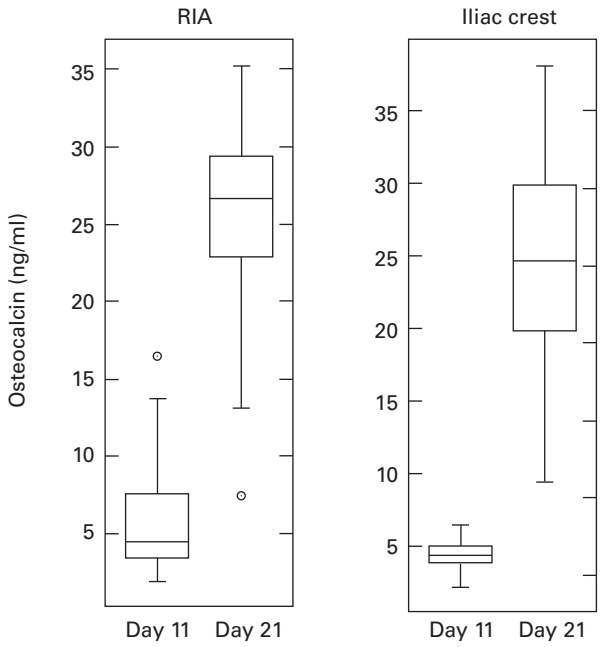

Fig. 2

Box plots showing the concentration of osteocalcin in pre- (day 11) and post-differentiation media (day 21) supernatants obtained from the Reamer–Irrigator–Aspirator (RIA) and iliac crest techniques. The boxes indicate the median and interquartile range (IQR), the whiskers denote the range of data and ° represents outliers (defined as ±1.5 × IQR).

Alizarin red staining. A duplicate flask for each sample was used for Alizarin red staining to assess calcium deposition, which indicates osteogenic potential.[22,23] A 5 ml aliquot of warm Alizarin red was added to a fully confluent cell layer in the 25 cm^2 flask. After five minutes of incubation the Alizarin was aspirated and the cells were washed three times with warm D-PBS. The retention of dye, indicating calcium deposition and osteogenic potential, was then evaluated microscopically by two authors (HSU and BEP).

Statistical analysis. This was performed using Student's *t*-test to compare the mean changes in the concentration of osteocalcin between days 11 and 21, as well as between RIA and iliac crest graft. A p-value ≤ 0.05 was considered to be significant. A chi-squared goodness-of-fit test was performed on the distribution of gender. Also, on the basis of obtained data a *post-hoc* power analysis was performed to determine how many specimens would be required to show a significant difference between RIA and iliac crest graft for the mean increase in osteocalcin. Statistical analysis was performed with StatPlus:mac (AnalystSoft Inc., Alexandria, Virginia).

Results

Patient demographics. Allocation to RIA or an iliac crest procedure was dependent on the preference of the surgeon. There were 25 patients in the RIA group and 21 in the iliac crest group. The demographics of the groups are given in Table I. The gender distribution was significantly different between the groups (p = 0.0209), with a higher proportion of women in the iliac crest group and a higher percentage of men in the RIA group, but there was no significant difference in age, tobacco or alcohol abuse or the prevalence of diabetes mellitus (Table I). Also there was no significant difference in the prevalence of other diseases known to be associated with the quality of bone, such as thyroid dysfunction, steroid usage, immunosuppression or osteoporosis.

Cell viability and FACS. All RIA and iliac crest samples had a cell viability of > 95% (Fig. 1). Flow cytometry performed on cells obtained on the day of surgery showed them to be

Table III. Pre- and post-differentiation concentration of osteocalcin in samples from the Reamer–Irrigator–Aspirator (RIA) and iliac crest techniques (CI, confidence interval; IQR, interquartile range)

	Osteocalcin concentration	
	Mean (ng/ml) (95% CI)	**Median (IQR)**
RIA (n = 34)		
Day 11	5.55 (4.092 to 7.017)	4.47 (2.74)
Day 21	25.85 (24.39 to 27.31)	26.6 (6.18)
p-value*	< 0.001	
Iliac crest (n = 22)		
Day 11	4.42 (2.392 to 6.448)	4.36 (1.11)
Day 21	24.68 (22.66 to 26.71)	24.7 (10.51)
p-value*	< 0.001	

* Student's *t*-test

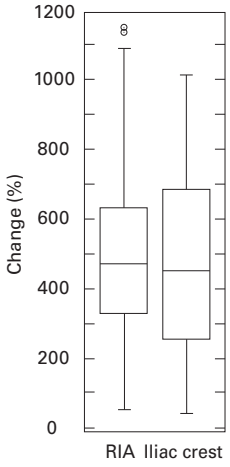

Fig. 3

Box plots showing the percentage change between pre- and post-differentiation in the Reamer–Irrigator–Aspirator (RIA) and iliac crest groups. The boxes indicate the median and interquartile range (IQR), the whiskers denote the range of data and ° represents outliers (defined as ±1.5 × IQR).

positive for CD34 and CD45 and negative for CD90 and CD105, indicating an initial cell population dominated by cells of haematopoietic lineage. After 28 days of culture the cells were negative for CD34 and CD45 and positive for CD90 and CD105, indicative of mature MSCs (Table II).

ELISA. The mean secretion of osteocalcin increased significantly in both groups (both $p < 0.001$) (Fig. 2, Table III). The osteocalcin concentration increased by a mean of 479.4% (95% confidence interval (CI) 393.3 to 621.9) in the RIA samples and by 507.6% (95% CI 387.5 to 571.4) in the iliac crest samples between days 11 and 21, with no significant difference between the groups ($p = 0.70$) (Fig. 3 and Table IV).

Morphological changes. Morphological changes similar to those observed by Wenisch et al[1] were also seen in the cell culture. Figure 4 demonstrates a typical morphological progression throughout the duration of culture. The cells began as small round cells and began to elongate and form sheets of dendritic like cells as they adhered to the flask.

Alizarin red staining. Cells stained with Alizarin red at day 28 of culture retained the red dye, indicating the presence of a calcium substrate within the cell layer in all samples (Fig. 5).

Table IV. Mean percentage change in osteocalcin concentration seen before and after differentiation in the Reamer–Irrigator–Aspirator (RIA) and iliac crest techniques (CI, confidence interval; IQR, interquartile range)

	Change in osteocalcin concentration	
	Mean (%) (95% CI)	**Median (IQR)**
RIA (n = 34)	479.44 (393.3 to 621.9)	471 (369.58)
Iliac crest (n = 22)	507.59 (387.5 to 571.4)	447 (283.84)
p-value*	0.70	

* Student's *t*-test

Discussion

We focused on quantifying the viability of the cells after reaming with RIA to further investigate the potential of these cells to mature into the osteoblastic lineage. The cells obtained using the RIA system were viable and, when cultured, matured into MSCs, which were osteogenic as confirmed by the production of osteocalcin and positive Alizarin red staining. There was no statistically significant difference in cell viability or osteogenic potential compared with graft harvested from the iliac crest.

Giannoudis et al[24] reported that reaming the intramedullary cavity of the femora produced an increase in systemic levels of growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β. The authors suggested that this increase, coupled with autograft deposition at the fracture site during reaming, may play a role in the healing of diaphyseal fractures treated by reaming.[24]

However, standard reaming is not without its drawbacks. It has been shown to increase medullary pressure sufficiently to cause marrow embolisation,[25] and also to generate sufficient heat to cause thermal necrosis of the bone.[26] In a sheep model, standard reaming was found to increase intramedullary pressures up to 600 mmHg, leading to bone marrow emboli surrounded by thrombotic aggregates.[27] The RIA was developed to reduce the likelihood of these phenomena.

To our knowledge, this is the first study to quantify the viability and osteogenic potential of medullary cells captured via the RIA system. Comparing the RIA effluent flow-through with iliac crest aspirate, Cox et al[16] found that the RIA effluent was a source of MSCs capable of differentiation into an osteogenic phenotype similar to those from the iliac crest in a small sample of patients (n = 6). The experimental design mimicked that of Porter et al,[15] who showed that the RIA effluent had the potential for osteogenic and

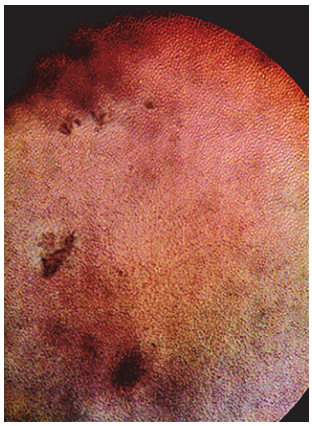

Fig. 4a

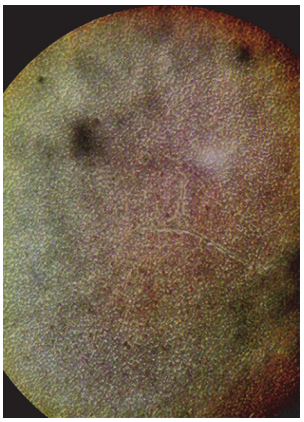

Fig. 4b

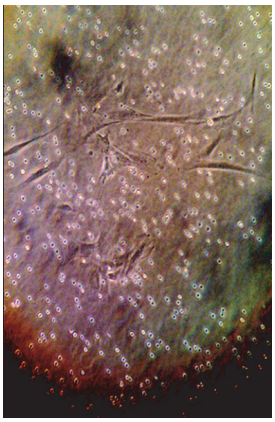

Fig. 4c

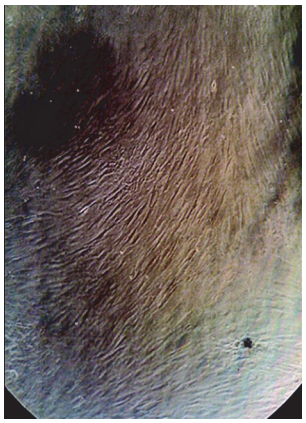

Fig. 4d

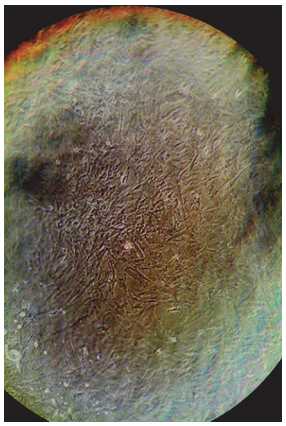

Fig. 4e

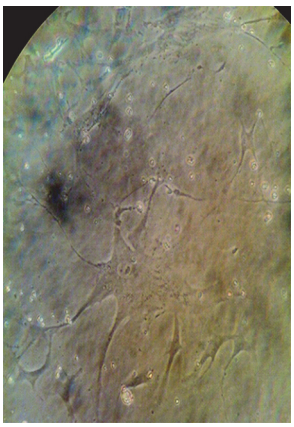

Fig. 4f

Culture sample images at 40× magnification showing typical morphological changes throughout the duration of the culture for both groups, at a) four days, b) seven days, c) 11 days, d) 14 days, e) 15 to 20 days, and f) 21 to 28 days. The cells began as small round cells and began to elongate and form sheets of dendritic like cells as they adhered to the flask.

chondrogenic differentiation. However, the viability of the cells was not assessed, nor was it assessed in Frolke et al's[2] study using standard reamers. Our study went one step further and looked at the filtered reaming debris in comparison with the iliac crest as a source of viable bone graft in a much larger patient population, and we demonstrated > 95% viability in the RIA group. Osteocalcin, the major non-collagenous protein found in mature bone matrix, was measured as an indicator of differentiation to signify osteogenic lineage. We found statistically significant increase in osteocalcin after induction with differentiating media for both RIA and iliac crest graft. All the cultures stained positive for calcium deposition, demonstrating further osteogenesis. Taken together, these results show that cells harvested with RIA retain osteogenic viability and the capacity to differentiate into mature osteogenic cells similar to graft harvested from the iliac crest.

There are limitations to this study. Patients were not randomised to each group, the type of bone grafting procedure was chosen by the surgeon and the numbers of patients in each group were not equal. We postulate that this difference is due to the indications, at surgery, for the choice of graft. Orthopaedic trauma patients, usually young men, are more likely to require large grafts that would require RIA. However, our results were consistent for each group independent of the source of bone graft. Also, we have shown that there is a high statistical significance with respect to osteocalcin production and viability within each group. Based on the results of this study and a *post hoc* power analysis, 367 samples per group would be required to reach a power of 0.8 to detect a significant difference

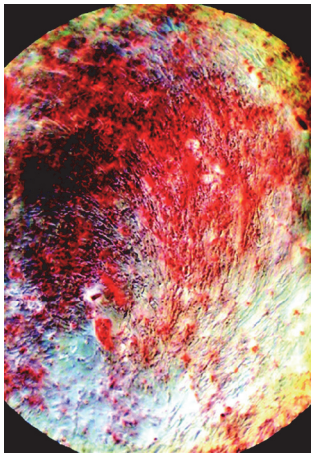

Fig. 5

Culture sample image at 40× magnification of Alizarin red S staining in a mature culture (at 28 days) showing retention of dye, indicating the presence of a calcium substrate within the cell layer.

between the groups. Using many techniques to assess viability and cell differentiation, our results were supportive of the hypothesis that cells harvested using the RIA system are viable and the graft material is capable of inducing MSCs toward an osteogenic lineage similarly to graft obtained from the iliac crest.

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