Seeding of Human Bone Marrow Stromal Cells onto Bone Graft Substitutes and Allograft - An Impaction Grafting Model

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Table of contents.

Abbreviations.

α–MEM – Minimum Essential Media – alpha modification.

AVN – Avascular Necrosis.

BMPs – Bone Morphogenetic Proteins.

β-TCP – Beta Tricalcium Phosphate.

CFMDA – 5 – Chloromethylfluorescein diacetate ("Cell Tracker Green")

CFU-F – Colony Forming Unit – Fibroblastic.

EDTA - Ethylenediamine tetra acetic acid .

EH - 1 - Ethidium Homodimer-1

FCS – Fetal Calf Serum.

HA – Hydroxyapatite.

HBMSC – Human Bone Marrow Stromal Cell.

MSC – Mesenchymal Stem Cell.

PBS – Phosphate Buffer Solution.

PLA – Polylactic Acid.

$ScC0_2$ – Supercritical Carbon Dioxide.

SCID mouse – Severe Combined Immunodeficient (nude) Mouse.

Abstract.

Aims.

This thesis examines the potential of Human Bone Marrow Stromal Cell (HBMSC) fractions to augment bone formation after impaction bone grafting. The project will examine the biological response of marrow derived cells on washed morsellised allograft, Beta – Tricalcium Phosphate (β-TCP) and poly(DL-lactic) acid (PLA) synthetic bone graft substitutes in an impaction grafting model.

Study Design.

Study I: In – vitro analysis of unselected HBMSC populations seeded onto natural (allograft) and synthetic (β-TCP and PLA) scaffolds after impaction.

Study II: Parallel in – vitro and in – vivo analysis of unselected and immunoselected HBMSC populations seeded onto natural (allograft) and synthetic (PLA and β-TCP) scaffolds after impaction.

Study III: Proof of concept clinical translation of methods from laboratory to operating theatre utilising autologous HBMSCs seeded onto washed allograft and impacted into two patients with proximal femoral defects.

Results.

Washed human bone allograft seeded with culture expanded HBMSCs from 4 patient donors, impacted and cultured *in – vitro* for 7 and 17 days. Cellular proliferation and increasing osteoblastic activity was observed between 7 and 17 days based on biochemical and live cell staining analysis.

Similar studies were performed with HBMSCs from 3 patient donors added to, and impacted on a PLA scaffold over the same time period at the same seeding density. Osteoblastic proliferation was also observed between 7 and 17 days.

Short term viability was also confirmed with cells from 3 patient donors seeded onto β-TCP graft substitute and impacted in an identical manner prior to proceeding to the next stage of the study.

28 day culture in-vitro and in vivo (SCID mouse model) of unselected and immunoselected cells seeded either on washed allograft, or β-TCP and impacted. Although both cell populations were viable on both grafts, the immunoselected cells would appear to be more successful in their proliferation especially when seeded onto β-TCP graft substitute.

Techniques in bone marrow aspiration combined with allograft preparation were employed to fill areas of proximal femoral bone loss in two patients, the first with a benign bone cyst and the second suffering from avascular necrosis (AVN). A live composite graft was produced in theatre and impacted into the patients, both of whom

demonstrated clinical and radiographic evidence of successful graft incorporation at follow-up.

Conclusions.

- HBMSCs seeded on washed allograft and synthetic PLA or β-TCP can survive and proliferate following the physiological insult of a standard femoral impaction.
- Immunoselected bone marrow progenitor cells seeded onto synthetic β-TCP scaffolds and impacted produce a more integrated and osteoid rich living composite than unselected HBMSCs on washed allograft.
- The techniques of bone marrow aspiration, seeding onto washed allograft and impaction can be adapted in a facile manner to the operating theatre to successfully reconstitute areas of extensive bone loss.

Publications, presentations, posters and abstracts.

Publications.

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Morgan S M, **Tilley S**, Perera S, Ellis M J, Kanczler J, Chaudhuri J B, Oreffo R O C.

Biomaterials, 2007 Dec;28(35):5332-43. Epub ahead of print 2007 Sep 5.

Adult mesenchymal stem cells and impaction grafting: a new clinical paradigm shift. (Review article)

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Taking tissue-engineering principles into theatre: augmentation of impacted allograft with human bone marrow stromal cells.

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Presentations.

Third Prize, British Orthopaedic Research Society (BORS) Annual Meeting.

Tilley S, Dunlop DG, Oreffo ROC. Synthetic Scaffolds Seeded with Mesenchymal Stem Cells - The Future of Impaction Bone Grafting. *Stanmore, July 2005.*

First Prize, Gauvain Society Meeting.

Tilley S, Dunlop DG, Oreffo ROC. Synthetic Scaffolds Seeded with Mesenchymal Stem Cells - The Future of Impaction Bone Grafting. *Basingstoke, June 2005.*

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Posters.

Translation from laboratory to theatre: augmentation of impacted allograft with human bone marrow stromal cells.

BJRF Bolland, **S Tilley**, K Partridge, AMR New, DG Dunlop, ROC Oreffo.

(BORS, Southampton July 2006, Research and Development Conference, Southampton University, April 2007).

First Prize, Clinical Science poster prize, Bone and Tooth Society (BATS) Annual Meeting.

Tilley S, Dunlop DG, Oreffo ROC. Synthetic Scaffolds Seeded with Mesenchymal Stem Cells - The Future of Impaction Bone Grafting.

Birmingham, July 2005.

Abstracts.

Improving both the biological and mechanical properties of allograft in impaction bone grafting. The potential role of human bone marrow stromal cells.

BJRF Bolland, K Partridge, **S Tilley**, AMR New, DG Dunlop, ROC Oreffo.

(Supps JBJS Br), (EFORT 2007).

Synthetic scaffolds seeded with mesenchymal stem cells – the future of impaction bone grafting?

S. Tilley, D. G. Dunlop, and R. O. C. Oreffo.

J Bone Joint Surg Br Orthopaedic Proceedings, 2006 88-B: 403.

Translation from laboratory to theatre: augmentation of impacted allograft with human bone marrow stromal cells.

BJRF Bolland, **S Tilley**, K Partridge, AMR New, DG Dunlop, ROC Oreffo.

(Supps JBJS Br), (BORS 2006).

Major Null-Hypothesis.

"Bone marrow stromal fractions seeded onto washed morsellised allograft or synthetic Beta Tricalcium Phosphate (β-TCP) and poly(DL-lactic) acid (PLA) graft substitute will not survive and proliferate with a bone phenotype after impaction grafting".

CHAPTER 1

1.1 Introduction.

The National Joint Registry reported 58,952 primary total hip replacements performed in England and Wales between 2006 and 2007(Drury P 2007). Widely regarded as one of the most successful advances in orthopaedic surgery, the incidence of primary THR has increased by 18% in the United Kingdom since 1991 and 2000 (Dixon, T. et al. 2004). Patient satisfaction is now over 96% at 30 years follow up (Wroblewski, B. M. et al. 2006) and component survivorship at the Exeter group is recorded at greater than 91% at 12 years (Williams, H. D. et al. 2002).

While these results are encouraging and are expected to improve with advances in components and surgical technique. In the UK revision rates more than doubled between 1991 and 2000 with 20% of all hip surgical procedures now representing revision work (Dixon, T. et al. 2004). Higher rates of primary hip arthroplasty were also observed in the United States during a similar time period (1990 -2002) (Kurtz, S. et al. 2005), the number of cases increasing by 50% over the thirteen years. Revision rates in the US were more comparable to the UK with a mean revision burden of 17.5%. The demand for revision surgery is projected to double by 2026 (Kurtz, S. et al. 2007) due to the increasing number of primary replacements which will ultimately fail by various mechanisms.

As implants fail, host bone is lost as a result of:

Aseptic loosening

Stress-shielding

Infection

Instability

Periprosthetic fracture

Aseptic osteolysis is the most common reason for implant failure and accounts for approximately 75% of cases (Holt, G. et al. 2007). This is a time dependent process which arises from an inflammatory reaction against polyethylene particulate debris. The particles exert their biological activity by the process of phagocytosis by macrophages. When particles, in particular those in the 0.1 – 0.5 μm range are phagocytosed in sufficient amounts the macrophages enter into an activated state of metabolism releasing substances that can result in periprosthetic bone resorption (Schmalzried, T. P. et al. 1999). Osteolysis on the acetabular side is generally asymptomatic and may occur with well fixed prostheses. On the femoral side wear debris migrates down the bone – cement (or press fit) interface leading to endosteal, intracortical, or non – linear bone destruction.

Stress - shielding involves redistribution of load when the femoral head is replaced by the femoral component of a total hip replacement. This has the effect of reducing stress on the proximal femoral cortex as the load is bypassed via the stem more distally. Consequently bone is lost as a result of a reduction in the load transmitted to bone. In addition, bone with a reduced density as a result of stress – shielding may be more susceptible to osteolysis.

Infection accounts for approximately 7% of patients requiring revision surgery. Revision procedures for infection are associated with a longer operative time, increased blood loss, and a higher complication rate compared with revisions for aseptic loosening (Bozic, K. J. et al. 2005b). The potential routes for transmission of bacteria are numerous and involve environmental, surgical and patient factors. Once inoculated, bacteria approach the prosthetic joint surface via a combination of physical and chemotactic factors. Being relatively inert the prosthesis provides no protection against colonisation (Gristina, A. G. et al. 1990). It would appear that specific bacteria have a particular affinity for certain bearing surfaces; *Staphylococcus aureus* for metal alloys and *Pseudomonas* for polymers. Successful management is dependent on removal of the prosthesis and all infected tissue. Re-implantation of a new prosthesis into a sterile bed is the goal and may be performed as a single stage or more commonly as a two stage procedure, separated by a period of local and systemic antibiotic delivery (Mitchell, P. A. et al. 2003).

Hip instability involves subluxation and dislocation and can present at any timepoint following arthroplasty. The aetiology is often multifactorial involving operative technique, patient factors and implant design. Key surgical risk factors include component malpositioning, decreased femoral offset and abductor insufficiency. Patient factors include older, female patients, those with neurological or cognitive dysfunction, inflammatory arthritis and those undergoing arthroplasty following femoral neck fracture (Morrey, B. F. 1992).

The incidence of periprosthetic femoral fractures is increasing due to a combination of increasing patient activity postoperatively and also as a direct consequence of the

increasing numbers of primary procedures being performed. The elderly are particularly vulnerable to low energy periprosthetic fractures due to osteopaenia or osteoporosis (Tsiridis, E. et al. 2007).Overall incidence is reported to range from 0.1% to 6% (Giannoudis, P. V. et al. 2007). Reconstruction options depend on the location of the fracture and whether the stem is well fixed or loose and range from nonoperative observation to customised massive endoprostheses augmented with allograft.

Defects on the acetabular and proximal femoral sides are classified using the American Academy of Orthopaedic Surgeons (AAOS) classification, described by D'Antonio et al (D'Antonio, J. et al. 1993) (Table 1).

Defect	Acetabulum	Proximal Part of Femur
Type I	Segmental. Region either peripheral or central (absent medial wall).	Segmental. Loss of proximal cortical integrity. Region in proximal third, intercalary, or in greater trochanter.
Type II	Cavitary. Preserved periphery and deformed, ballooned socket.	Cavitary. Contained proximal femoral defect with gross cancellous bone loss and cortical ectasia without cortical breech.
Type III	Combined I and II.	Combined I and II.
Type IV	Pelvic discontinuity. Dissociation among ileum, ischium and pubis.	Malalignment. Often sequel to periprosthetic fracture. Rotational or angular deformity results.
Type V	Arthrodesis. Previous disease or surgery obliterating acetabulum	Stenosis. Characterised by intraluminal narrowing.
Type VI		Discontinuity. Proximal and distal parts of femoral shaft dissociated. Usually periprosthetic defect.

Table 1. Classification of defects in the hip according to the AAOS.

Anaesthetic and surgical techniques continue to improve allowing older patients and those with associated co-morbidity to be increasingly considered for arthroplasty surgery. At the opposite end of the spectrum a younger and more active patient group will ultimately live longer and place higher demands on their joint replacements (Toms, A. D. et al. 2004).

Revision hip surgery is economically costly in terms of a longer hospital stay, more expensive components, the need for experienced staff and often, extended follow-up. Physiologically the procedure is also challenging compared to primary THR with a

longer operative time, increased blood loss and a higher post-operative complication rate (Bozic, K. J. et al. 2005a).

In addressing the often substantial regions of bone loss seen in revision hip surgery the surgeon can now either bypass the deficient area with large prostheses or make an attempt at reconstituting the deficient areas with bone graft prior to inserting a new prosthesis. When considering replacement of lost bone stock, autologous bone is the graft of choice as it contains osteogenic cells within the marrow, an osteoconductive collagen matrix as well as Osteoinductive proteins and factors endogenous to bone. However in the case of revision hip surgery the degree of lost bone is often so significant that the use of autograft would be associated with unacceptable donor site morbidity.

To address this clinical need, cancellous bone impaction grafting has proven a robust method of replacing lost bone in the acetabulum and proximal femur in these patients. The aim of the procedure is to achieve stability of the implant with the use of compacted, morsellised graft which will eventually allow the restoration of living bone stock around the revised prosthesis by bone ingrowth such that it resembles the primary replacement.

Acetabular reconstruction is achieved by progressive layering of impacted cancellous bone into cavity defects and on the femoral side a neo-endosteum is produced by layered impaction into the femoral canal of the same morsellised cancellous allograft. The allograft is supplied as fresh frozen donated femoral heads from regional bone banks. The potential for disease transmission remains the largest problem in bone

banking (Aspenberg, P. 1998) and each donated femoral head is screened for Hepatitis B surface antigen and core antibody, Hepatitis C antibodies, Human Immunodeficiency Virus 1 and 2 antibodies and Syphilis. Despite stringent donor testing there are reports of both graft contamination detected after implantation into the recipient and also disease transmission following initially negative screening (Barriga, A. et al. 2004;Kainer, M. A. et al. 2004).

The original graft preparation and impaction technique was first described by Sloof (Slooff, T. J. et al. 1984) using morsellised cancellous bone. Although the technique has evolved with respect to technique and instrumentation the graft type and preparation has remained constant. The initial argument for using cancellous graft was that the open structure would allow for more rapid angiogenesis and osteoclast driven remodelling (Goldberg, V. M. 2000). This mechanism has been called into question with favourable results being seen using morsellised cortical allograft (Kligman, M. et al. 2003).

Studies by Dunlop and Brewster have confirmed that a well graded mix of highly washed allograft particles provided optimal initial mechanical stability (Dunlop, D. G. et al. 2003;Brewster, N. T. et al. 1999). The grading of different particle sizes based on soil mechanics principles allows interdigitation of graft particles of various sizes. This has direct advantages in resisting shear within the graft. Critically particle sizes between 3 and 5 mm on the femoral side and larger at 8 to 10 mm on the acetabular side were felt to provide the most stable construct (Fosse, L. et al. 2006). Clinically this is easy to perform with bone mills providing the smaller particles and bone nibblers producing the larger "croutons".

Following graft preparation, it is then impacted around a "phantom" femoral component. This stage is critical to the success of the procedure and judging the appropriate degree of compaction is often difficult especially in the presence of a thin cortex. The process of graft compaction causes plastic deformation of the particles allowing them to assume a new structure of smaller volume and strong enough to support a cemented prosthesis. Appropriate compaction is essential in preventing early prosthesis subsidence but overly vigorous impaction may lead to intraoperative femoral fracture, a recognised complication of the procedure. Cement is then pressurised into the newly formed canal and the prosthesis is introduced in a similar fashion to primary hip arthroplasty. Cement is in direct contact with the impacted graft layer, which immediately becomes a load-bearing material (Bavadekar, A. et al. 2001). Typically the patient will be partially weight bearing on the operated limb for six weeks as the graft stabilises with fibrous ingrowth and variable amounts of incorporation.

Although still in its infancy, first reported in 1991 (Simon J-P 1991), the process of femoral impaction grafting has evolved rapidly with advances in surgical technique and the development of dedicated instrumentation. As a result, complications such as excessive femoral subsidence and diaphyseal femoral fracture have been reduced. The degree of acetabular and proximal femoral bone stock loss in patients undergoing revision hip arthroplasty is highly variable as is the surrounding soft tissues and the physiological state of the patient. It is therefore not surprising that there is significant variation in survivorship after impaction grafting procedures. Recent studies have detailed results not dissimilar to primary arthroplasty with survivorship now reported between 99% to 100% at 10 to 11 years using femoral reoperation for symptomatic

aseptic loosening as the endpoint (Halliday, B. R. et al. 2003;Schreurs, B. W. et al. 2005). However, other studies report much lower success rates (Jazrawi, L. M. et al. 1999), in particular involving acetabular reconstruction without the use of cement (Jeffery, M. et al. 2003). Board and colleagues (Board, T. N. et al. 2006) attributed this wide variation in reported survival to several factors including the aetiology of failure of the primary THR, degree of osteolysis, implant choice, surgical technique and bone graft considerations.

Despite good functional and radiographic results, histological analysis of biopsy specimens and retrieved specimens remains varied. Early histological reports (Ling, R. S. et al. 1993) described allograft being largely replaced by living cortical bone over 90% of the total surface area of the sections studied. It was noted that the interface between the cement and tissue resembled that seen after a primary THR. Subsequent retrieval studies present less predictable results (Linder, L. 2000;Nelissen, R. G. et al. 1995). Histological evaluation of a retrieved specimen by Weidenhielm *et al* (Weidenhielm, L. R. et al. 2001) again showed variable graft incorporation 3 years after revision arthroplasty using impaction bone grafting. This paper identified 3 ill-defined zones:

1) Inner zone containing bone cement, vascularised fibrous tissue and fragments of necrotic bone graft and partially necrotic trabeculae.

2) Middle zone of predominantly viable trabecular bone and an incomplete peripheral "neo-cortex".

3) Outer zone viable cortex.

Histology from recovered specimens would suggest that there is a slow in- and on-growth of new bone onto the surfaces of the allograft granules, with a very slow rate of graft resorption (Sorensen, J. et al. 2003). The literature would suggest that the strength of the composite is the result of a combination of bony ingrowth and fibrous armouring. The former may be encouraged by osteoinduction form the morsellisation process which increases the graft surface area and releases growth factors and osteoconduction with the graft acting as a scaffold. The third determinant noted by Toms et al (Toms, A. D. et al. 2004) and currently generating significant interest in the literature is the biological effect of mechanical loading which produces plastic deformation and stimulates bone formation. Indeed Board et al has shown that BMP-7 is released from fresh-frozen cancellous allograft in proportion to the strain applied to the bone (Board, T. N. et al. 2008). This suggests that the impaction process itself may contribute to the biological process of remodelling and bony incorporation.

Fibrous tissue ingrowth seems to precede bone formation. Indeed, the mechanical properties of the composite are enhanced by "armouring" with ingrowing fibrous tissue (Tagil, M. et al. 2001). This initial load bearing composite may be stronger than that of allograft alone. More recently, Positron Emission Tomography (PET) studies in 5 patients followed up over 6 years demonstrated bone formation throughout the graft in the first year. The initially increased bone metabolism largely normalised at 6 years except distal to the stem tip and small localised areas proximally which were felt to represent fibrous healing (Ullmark, G. et al. 2007). This heterogeneous picture of a mixture of living and dead bone would indicate that complete osseous remodelling may not be necessary to obtain a good clinical result. Given that compacted allograft is then sandwiched between the inhospitable layers of denuded

cortical bone and the cement mantle it is impressive that the graft is able to revascularise, remodel and eventually restore lost bone stock.

Attention recently has focussed on the use of bone graft extenders to improve mechanical stability and reduce the reliance on donated femoral heads. Ceramics such as BoneSave (80% tricalcium phosphate / 20% hydroxyapatite) has been evaluated by Blom et al in an impaction grafting model of the femur in four groups of sheep with varying quantities of BoneSave and allograft (Blom, A. W. et al. 2005). Group one received pure allograft, group two 50% allograft and 50% BoneSave, group three 50% allograft and 50% BoneSave and group four 10% allograft and 90% BoneSave as the graft material. Function was assessed using pre- and post-operative ground reaction forces. Bone mineral density was also compared using dual energy X ray absorptiometry (DEXA) scanning. Loosening and subsidence were assessed radiographically and histologically in the explanted specimens. There was no statistically significant difference between the four groups after 18 months of unrestricted functional loading for all outcome measures.

A similar TCP/HA bone-graft extender was evaluated by (van Haaren, E. H. et al. 2005) using a human cadaveric impaction grafting model. The study concluded that TCP/HA allograft mix increased the risk of producing a fissure in the femur during the impaction procedure, but provided a higher initial mechanical stability when compared with bone graft alone.

At the microscopic level porosity and pore size of ceramic scaffolds play a critical role in bone formation in vitro and in vivo. In vitro, lower porosity stimulates osteogenesis by suppressing cell proliferation and forcing cell aggregation. In

contrast, in vivo, higher porosity and pore size result in greater bone ingrowth (Karageorgiou, V. et al. 2005).

It is generally accepted that the minimum requirement for pore size is approximately 100 μm, however, pore sizes >300 μm are preferred due to superior osteogenesis and capillary formation (Jones, A. C. et al. 2007). Pore size has been shown to affect osteogenesis; smaller pores favoring hypoxic conditions and inducing osteochondral formation before osteogenesis, while large pores, that are well-vascularized, lead to direct osteogenesis without preceding cartilage formation (Karageorgiou, V. et al. 2005).

Ceramic materials such as HA and TCP appear to aid initial mechanical stability but little is known about their biological activity in supporting osteogenic stromal cells both in vitro and in vivo (Kasten, P. et al. 2008) especially under conditions of impaction. This thesis will address these issues and also examines newer graft substitutes with similar mechanical characteristics to cancellous bone.

Due to the rapidly increasing number of applications, demand for bone allograft in general continues to outstrip supply (Hing, K. A. 2004) and, specifically, in the field of revision THR the greater need for femoral heads has not been matched with an increased supply (Galea, G. et al. 1998). It is therefore important that the allograft scaffold achieves adequate initial stability followed by the biological response of graft incorporation and remodelling (Board, T. N. et al. 2006). We propose that following the initial graft introduction the biological response can be augmented using tissue-engineering techniques.

The potential for tissue regeneration using cells of the marrow stromal system has generated huge interest in recent years. Bone marrow acts as a reservoir for both non-adherent, circulating haematopoietic cells in addition to marrow stromal cells (Figure 1).

Friedenstein and colleagues first described a rare (1 in $10 - 10^5$ mononuclear cells) population of spindle-shaped stromal cells present in the bone marrow that could proliferate to form colonies. These human bone marrow stromal cells (HBMSCs), under specific in vitro conditions possessed the ability to form colonies derived from a single cell. A sub-population of these fibroblastic colony forming units (CFU-Fs) could be made to differentiate along multiple mesodermal lineages to generate cartilage, bone, myelosupportive stroma, adipocytes and fibrous connective tissue (Friedenstein, A. J. et al. 1968;Friedenstein, A. J. 1976;Friedenstein, A. J. et al. 1978). The original assays required the bone marrow stromal cells to adhere to tissue culture plastic and since then the cells have been well characterised with regard to phenotype, plasticity and potentiality.

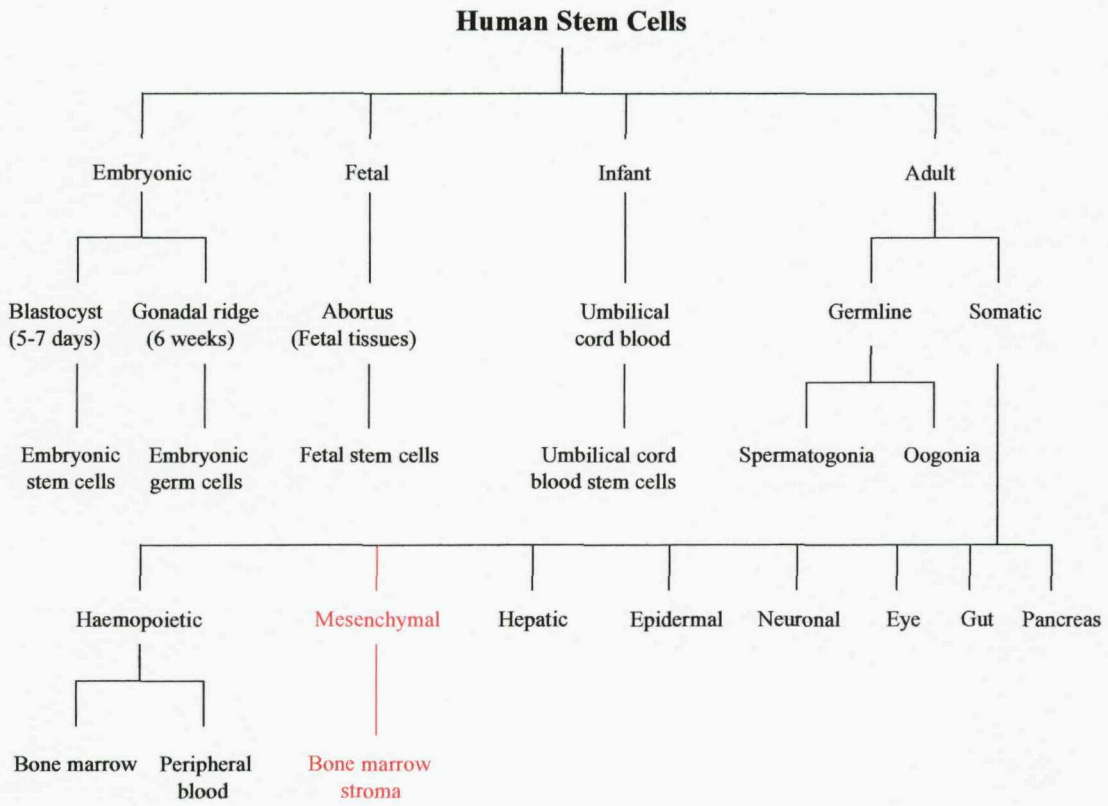


Figure 1. Differentiation pathways of Human Stem Cells. Adapted from (Lee, E. H. et al. 2006).

These HBMSCs are also referred to as osteogenic stem cells, marrow stromal fibroblastic cells, bone marrow stromal cells, stromal precursor cells and latterly mesenchymal stem cells.

The term "stem cell" can be applied to a diverse group of cells, which, regardless of their source characteristically fulfil the following criteria criteria:

(1) Possess capacity for self renewal.

(2) Have the ability to differentiate into more than one cell type.

(3) Maintain the capacity for cell division throughout life (Lajtha, L. G. 1979).

To date the isolation of a demonstrably pure stem cell fraction from the bone marrow stroma has proved elusive (Dawson, J. I. et al. 2008).

Mesenchymal Stem Cells (MSCs) have the ability to differentiate into more than one, but not all cell types and are thus termed multipotent, rather than pluripotent. They divide by asymmetric division, which produces an identical daughter cell in addition to a second progenitor cell. After appropriate stimulation the progenitor cell, lacking the capacity for self-renewal, becomes committed to a lineage-specific differentiation program (Figure 2). They are characterised by cell surface markers and can be induced to differentiate into musculoskeletal tissues such as bone, cartilage, tendon, ligament, skeletal muscle and fat (Rose, F. R. et al. 2002).

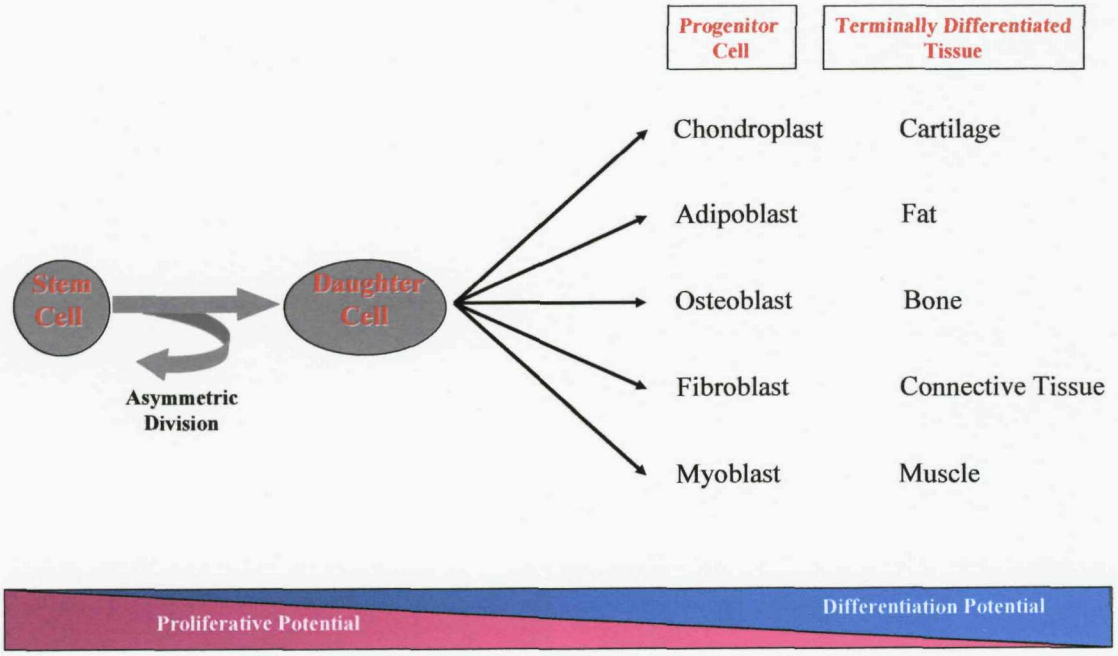


Figure 2. Differentiation of mesenchymal stem cells from self renewing stem cells (Adapted from (Oreffo ROC, Cooper C Mason C 2005).

Osteogenesis is triggered by the transition of MSCs into bone forming osteoblast cells and subsequent differentiation of these cells is exemplified by the synthesis, deposition and mineralization of extracellular matrix (Huang, W. et al. 2007). This process is tightly controlled and highly orchestrated (Song, L. et al. 2005), commitment and differentiation of the osteoblast under the complex control of signal transduction and transcriptional regulation of gene expression pathways.

Bone marrow aspirate is considered to be the most accessible and enriched source of MSCs and can be considered as the source of a common pool of multipotent cells which gain access to their target tissues via the circulation (Tuan, R. S. et al. 2003). The process of aspiration is a straightforward, safe clinical procedure which is often performed in the out-patient setting under local anaesthesia. Critically, the manipulation of adult marrow cells for autologous use avoids the ethical controversies of embryonic stem cell manipulation and offers the possibility of cellular regeneration over repair.

Recent studies have identified both non-adherent cells with osteogenic potential and similar cells with osteogenic potential in physiologically significant numbers in the peripheral circulation (Khosla, S. et al. 2006). The concentration of these peripheral (1 to 2% of mononuclear cells) and non-adherent cells increase during times of increased bone formation and can form bone in vivo (Eghbali-Fatourechi, G. Z. et al. 2005). Advances in our knowledge of these peripheral and non-adherent osteogenic progenitors will allow for easier and more efficient isolation and expansion and ultimately the potential for therapies via systemic infusion (Cancedda, R. et al. 2003).

When considering large bone reconstitution as is necessary for revision hip surgery, tissue engineered alternatives would have to demonstrate success with respect to:

1. Osteogenicity
2. Osteoinduction
3. Osteoconduction
4. Mechanical stimulation.
5. Biocompatability

Osteogenesis is the ability of the osteoblasts at the site of new bone formation to produce minerals to calcify the collagen matrix and form a new bony substrate. Osteoinductive factors induce the progression of mesenchymal stem cells and other osteoprogenitor cells towards the osteogenic lineage. An osteoconductive matrix will act as a scaffold into which cells attach and proliferate eventually leading to replacement and remodelling of the composite. Mechanical stimulation may also be critical in the proliferation of osteogenic cells and for the formation of bone mineral and structure (Wiesmann, H. P. et al. 2004). The composite must be biocompatible with the surrounding tissue displaying a lack of immunogenic response.

Studies have demonstrated cellular viability of MSCs implanted onto natural and synthetic supports (Kotobuki, N. et al. 2005;Abukawa, H. et al. 2004;Chen, F. et al. 2002;Cinotti, G. et al. 2004;Gao, J. et al. 2001;Livingston, T. L. et al. 2003). More recently a study by Mushipe have confirmed that MG63 cells from a human osteosarcoma line seeded onto morsellised bovine allograft survive a limited number of impactions in an acetabular impaction grafting model (Mushipe, M. T. et al. 2006). The cultured cells were seeded onto the allograft scaffold and impacted into an

acetabular cup model using a mechanical device constructed from data obtained during impaction grafting by an orthopaedic surgeon. After release of cells from the scaffold using trypsin the cells were assessed for viability using the double-stranded DNA PicoGreen® assay. Significant reductions in viable cell numbers were observed between the fifth impact as compared to the first and second impactions ($p<0.01$ and $p<0.05$ respectively). Cell survival rates dropped to 21.5% after the fifth impact. These findings were important in that they confirmed a significant degradation in cell numbers after relatively few impactions but the study did not address survival after much higher numbers of impactions as is the case with a standard IBG procedure.

Critical to IBG success is the range of forces that the graft is subjected to. The technique is highly operator dependant and the forces imparted on the graft vary considerably between surgeons. To this end Korda *et al* confirmed the viability of sheep mononuclear cells seeded onto allograft after impaction with a range of forces (Korda, M. et al. 2006). The cells were isolated and cultured under basal (without osteogenic stimulus) conditions. After seeding and agitating for 2.5 hours the composites was incubated for 72 hours prior to impaction. Data obtained from various surgeons was used to produce a range of impaction forces to be investigated (0, 3, 6 and 9 kN). The impaction force was regulated by the drop height of a 1 kg weight. Cell viability post impaction was determined using the Almar Blue assay. Cells remained viable in the 3 and 6 kN groups as evidenced by increasing absorbance of Almar Blue. Viability was seen to be reduced after the 9 kN force. The authors concluded that the addition of marrow stromal cells to allograft could survive normal impaction forces found in revision THR but recommended against excessively high impaction forces.

Deakin *et al* (Deakin, D. E. et al. 2007), concerned about the reduced rates of bone incorporation when using irradiated allograft chips reported improved results when augmenting this type of graft with autogenous marrow. The irradiated graft was washed to remove fat and then added to the marrow aspirated from the ipsilateral iliac crest to produce a 40% allograft-autologous marrow mixture. This elegant study involving 103 consecutive patients demonstrated evidence of graft cortication and trabeculation over a mean period of 44 months in over 90% of patients both on the femoral and acetabular sides. These results compares favourably with previously reported levels of incorporation of 40% when using irradiated bone alone which has virtually no inductive capacity. Despite the encouraging results there are concerns regarding initial strength and impact energy absorption of irradiated allograft, both of which are known to be significantly reduced even after low dose ionizing radiation (Currey, J. D. et al. 1997).

This study represented a paradigm shift with regards to graft preparation in IBG. Clinically it would appear that with the use of a live composite (graft augmented with autologous cells) was advantageous. This thesis examines the natural evolution of this study employing the advantages of un-irradiated graft and graft substitutes augmented with human bone marrow stromal fractions both clinically and at the microscopic level.

The isolation and enrichment of MSCs capable of being encouraged down the osteogenic pathway has been facilitated by the identification of a number of antibodies including the STRO-1 monoclonal antibody (Simmons, P. J. et al. 1991;Simmons, P. J. et al. 1994). The monoclonal STRO-1 IgM antibody was the first

antibody to be produced that could identify the CFU-F in adult bone marrow. The STRO-1 positive fraction of human bone marrow derived cells was elegantly shown to be capable of differentiating into multiple mesenchymal phenotypes given the appropriate stimuli (Dennis, J. E. et al. 2002). It recognises a trypsin–resistant cell surface antigen present on a subpopulation of human bone marrow derived colony forming cells (Stewart, K. et al. 1999) and has been used to demonstrate that the STRO-1^+ population of human bone marrow cells is capable of osteogenic differentiation (Gronthos, S. et al. 1994). The STRO-1 epitope however remains elusive although expression of STRO-1 is known to be limited to a fraction of bone marrow that contains osteogenic precursors and does not bind to macrophages, myeloid cells or megakaryocytes (Dennis, J. E. et al. 2002)

Our knowledge of the behaviour of human trabecular bone derived cells which express the STRO-1 antigen in rapidly increasing. In culture the cells display stem cell-like qualities characterised by a stable undifferentiated phenotype as well as the ability to proliferate extensively while retaining the potential to differentiate along multiple lineages (Tuli, R. et al. 2003). In addition, cells expressing the STRO-1 antigen have been shown to migrate out of their native bone niche to generate multipotential stem and progenitor cells (Song, L. et al. 2005). Clinically this characteristic of exiting the trabecular niche and localising to sites of wound healing and areas requiring new bone growth is vital to the success of bone grafting. The functional differentiation of cells expressing an osteogenic phenotype may be key to the development of a living graft composite rather than fibrous tissue armouring of dead cancellous bone graft.

In this project human bone marrow osteoprogenitors expressing the STRO-1 antigen were identified and specifically isolated using magnetic activated cell sorting (MACS). This technique has become the standard method for cell separation providing efficient enrichment of osteoprogenitors with yields of up to 100% of the STRO-1^+ population (Encina, N. R. et al. 1999).

The technique is based on superparamagnetic particles approximately 50nm in diameter coated with a mouse monoclonal antibody (STRO-1) that recognises a stromal cell surface protein. The beads are composed of a biodegradable iron oxide and polysaccharide matrix which typically disappear after a few days in culture. The labelling process does not appear to alter the structure, function or activity status of tagged cells(Miltenyi, S. et al. 1990). When a column is placed in the magnetic separator a high-gradient magnetic field is induced in the column matrix which effectively retains cells labelled with the magnetic beads. Unlabelled cells can pass through (STRO -1 negative fraction). The STRO-1 labelled cells (STRO-1 positive fraction) are released after removal of the column from the magnet (Figure 3).

Direct monoclonal antibody magnetic cell labelling was used in this project. It is fast and requires only one labelling step as the STRO-1 antibody is directly coupled to the magnetic particle. This avoids unnecessary cell washing and consequently cell loss.

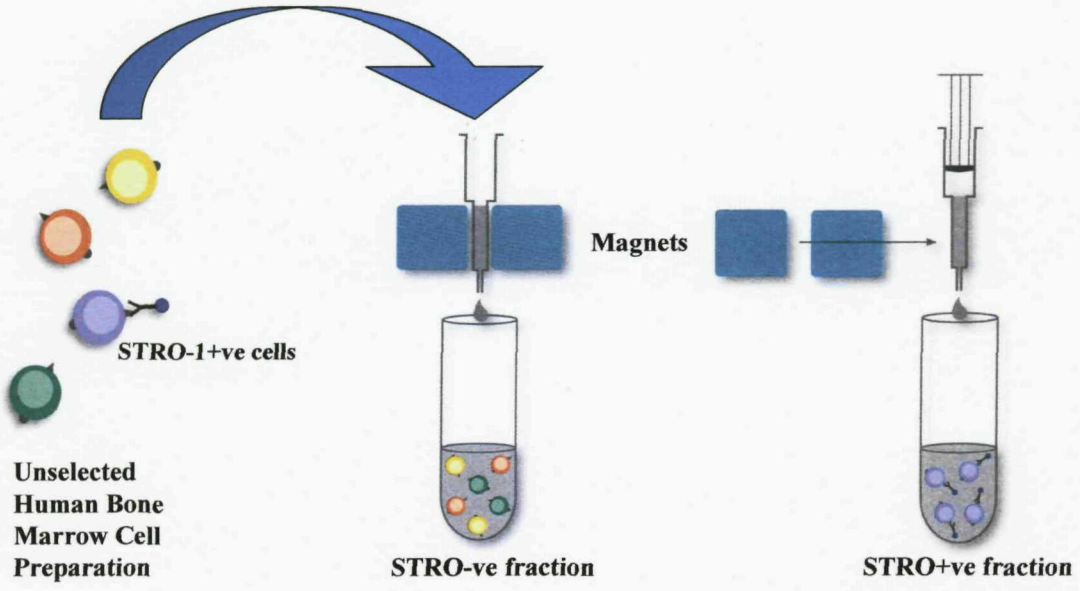


Figure 3. MACS selection of cells expressing the STRO-1$^+$ antigen. Adaption of original figure kindly donated by Dr Tare (Bone and Joint Research Group).

CHAPTER 2

2.1 Aims and objectives.

This thesis proposes a biological space filling model with autologous cells seeded onto both natural and synthetic scaffolds with the same mechanical properties as native proximal femoral endosteal bone. This composite would regenerate and remodel. We propose an algorithm (Figure 4) involving pre-operative bone marrow aspiration, ex vivo expansion of osteoprogenitor cells which are then seeded on a suitable scaffold creating a living composite. This is then impacted into the patient in the standard fashion. The strength to support a prosthesis would be a result of homogeneous periprosthetic bone formation and not reliant on fibrous tissue armouring. This method of bone stock restoration would also be eminently suitable for spinal (Li, H. et al. 2004) and selected benign tumour / cyst filling, trauma and revision knee surgery.

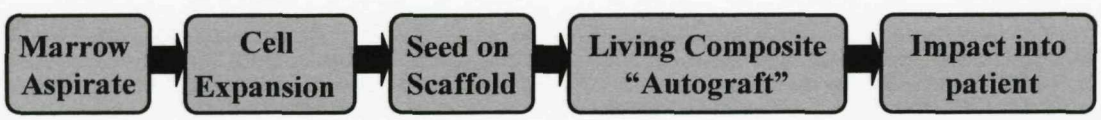


Figure 4. Schematic of proposed technique as applied to the surgical patient.

The studies described in this project reflect an evolution in techniques gained in the fields of tissue engineering, biochemistry and histology during the period of research. The studies examined involve classical tissue engineering techniques using organ – specific (osteogenic) cells seeded onto natural and synthetic scaffolds. Highly washed cancellous bone allograft was the natural scaffold and Beta Tri-calcium Phosphate (β-

TCP) and poly(DL-lactic) acid (PLA) graft substitute were used as the synthetic scaffolds in this study. Both marrow cell preparations and immunoselected progenitor cells containing a higher proportion of mesenchymal stem cells than are found in the native marrow were evaluated on both scaffolds.

2.2 Study limitations and failures

This study was undertaken with the aim of early clinical translation to the operating theatre. Although employing best practices of basic science techniques, it must be appreciated that variations in technique are required to this end. All aspects of the study were performed with an understanding of the potential clinical limitations. Examples of this include cell culture time prior to implantation. It was decided that no cell culture should progress beyond 14 days and passage 1 (P1) prior to seeding of the cells onto scaffolds. This was felt to reflect an appropriate amount of time from preoperative patient assessment and marrow aspiration to surgery. Using this end-point for cell expansion provided valuable information with regard to potential cell numbers after a suitable marrow aspiration. Conversely, direct comparisons between techniques were sometimes not possible due to differing cell numbers, a significant disadvantage in basic science but an everyday reality in surgery.

Human bone marrow samples were taken from a donor pool which included patients undergoing both elective total hip replacement for osteoarthritis and those who had suffered a traumatic fractured neck of femur and were receiving a hemiarthroplasty (femoral side only half hip replacement). When the author had performed or was involved in the operation and marrow collection the sex, age and pathology (osteoarthritis or osteoporosis) was recorded as documented in study 2. In many cases

only the age and sex was provided. This is potentially important in that the normal adult marrow stem cell populations vary very little in CFU-F forming potential (Oreffo, R. O. et al. 1998), however patients with advanced osteoarthritis see a reduction in the differentiation of their stem cells (Murphy, J. M. et al. 2002). Additionally patients who have suffered a fractured neck of femur are likely to have a local up - regulation of their stem cell numbers at the fracture site.

All cell cultures were carried out with Penicillin / Streptomycin antibiotic cover. Although largely successful, one major infection destroyed the cell lines that were destined for seeding onto the β-TCP scaffold in Study I. As a result, a differing seeding density had to be used over a shorter time period resulting in a less powerful comparison with the other scaffolds.

Histological preparation of the samples, in particular involving β-TCP and PLA was universally difficult. The friable nature of β-TCP meant that, even with various types of methacrylate embedding under high vacuum the specimens tended to crumble and disintegrate as the microtome blade passed through. The ultrastructure of PLA on the other hand tended to deform and plasticise after standard alcoholic fixation. This necessitated shorter fixation times and a protocol change in sample preparation to preserve the scaffold architecture (see Appendix 1 – laboratory techniques). These features reduced the efficiency of production of usable slide specimens to less than 5%.

It was hoped that all human bone marrow stromal cells which had been implanted into nude mice would be identifiable with a fluorescent tracker, the aim being to avoid

confusion with mouse cells at post mortem. Various reagents which freely diffused through the membranes of live cells were trialled but none managed to exhibit any fluorescence at 28 days and this was abandoned. An alternative assay using the supposedly human specific immunohistochemical Vimentin stain to isolate human from mouse cells was also examined but this also failed to accurately differentiate between the two. These findings were disappointing and as a result much of the analysis from in vivo culture was based on cellular morphological changes.

The original study timeline had allowed for a large animal study specifically to look at radiographic migration, mechanical competence and biological integration. Designed with 3 arms and to run over 12 weeks, power calculations, α and β errors were performed and it was hoped to carry out the study on 40 animals. Unfortunately licensing issues and lack of pen space due to another large animal study prevented this from being performed (see Appendix 4 – Large animal study proposal).

CHAPTER 3

Study I

In vitro analysis of marrow stromal cells seeded onto natural (allograft) and synthetic (PLA and β-TCP) bone grafts with impaction.

3.1 Introduction.

This study examines the medium and long-term survivability of culture-expanded human bone marrow stromal cells grown under osteogenic conditions and seeded onto washed morsellised human cancellous allograft and synthetic graft substitutes in an impaction grafting model. Provided the seeded cells survived the impaction process it was hypothesised that this method of graft production would be superior to standard bone grafting which utilises dead bone which provides a scaffold but does not directly contribute to osteogenesis (Burwell, R. G. 1985). The living composite grafts were subjected to forces and number of impactions equivalent to those experienced by washed allograft alone during a human impaction grafting procedure. Biocompatibility and maintenance of osteogenic behaviour was determined after 7 and 17 days culture *in–vitro*. Initial success using washed allograft prompted similar studies with poly(DL-lactic) acid (PLA) and β – tricalcium phosphate (β-TCP).

3.2 Materials and methods.

Reagents, Hardware and Software.

All tissue culture reagents including α–MEM, Fetal Calf Serum (FCS) and staining solutions were purchased from Sigma – Aldrich, UK unless otherwise stated. Cell Tracker Green™ CMFDA (5–chloromethyl–fluorescein diacetate) and Ethidium Homodimer–1 were purchased from Invitrogen, Paisley, UK. Polyethylene cylinders for containment of the impacted grafts were purchased from TAAB Laboratories Equipment Ltd (C094). Initially designed as embedding capsules, these 8mm diameter capsules were cut to 10mm in length to make a cylinder closed at one end and 18 perforations made with a 2.7mm drill (Figure 5). This allowed free flow of the culture media over the graft material. The size chosen allowed multiple separate cultures to be performed simultaneously, all studies being at least n=3. Additionally, cylinders of this size were thought suitable for subcutaneous implantation into SCID mice in Study II of the study. End caps were then reapplied to maintain the graft in its impacted state. All capsules were sterilised for 24 hours in 70% Ethanol and washed three times in sterile Phosphate Buffer Solution (PBS) prior to use.

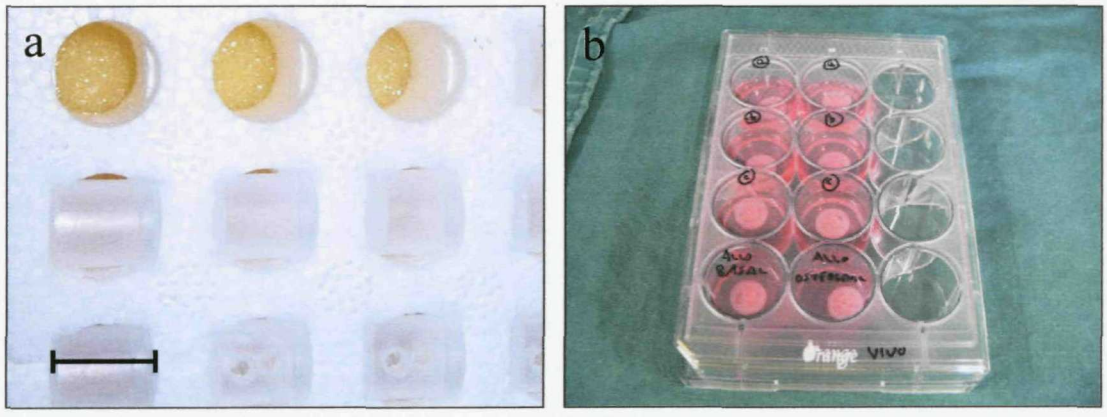


Figure 5. (a) Polyethylene cylinders before drilling (top row) and after drilling (bottom row), scale bar = 10mm. (b) Cylinders containing impacted graft in culture.

Isolation and Expansion of Human Bone Marrow Stromal Cells.

Human bone–marrow samples were retrieved with consent from haematologically normal patients undergoing elective and traumatic hip surgery at Southampton University Hospitals NHS Trust, in accordance with the Southampton and South West Hampshire Local Research Ethics Committee guidelines (LREC number199/94).

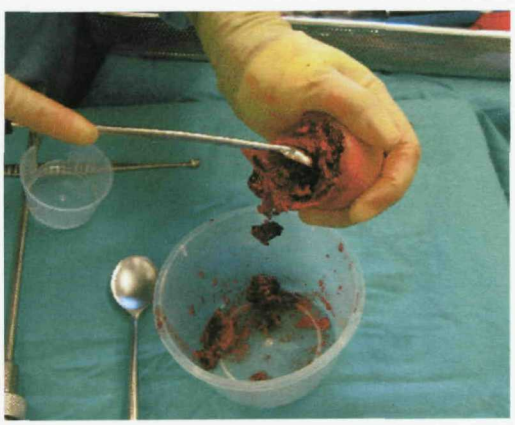


Figure 6. Curettage of retrieved femoral head prior to isolation of mononuclear cells.

After retrieval of the femoral head in theatre (Figure 6) marrow preparation was performed within 24 hours after overnight incubation at 4°C.

Isolation of marrow mononuclear cells was performed by repeatedly washing the marrow in filter sterilised α–MEM, removing the washed stromal cells prior to centrifugation at 1100rpm and 4°C for 5 minutes. The resultant pellet was resuspended in α-MEM after discarding the fatty layer, filtered with a 70μm filter to eliminate remaining bone debris and cell clumps, counted with a haemocytometer and plated immediately onto T150 flasks. The cells were cultured in α–MEM and 10% FCS under osteogenic conditions (50 μg/mL ascorbic acid and 100 nmol/L dexamethasone) repeated at each media change. Penicillin–streptomycin (100 mg/500 mL) was added to the media from the outset in an attempt to prevent culture infection. All studies were performed with passage 1 cells (P1). Cells were cultured in flasks at 37°C with 5% CO_2 to confluence and released using trypsin 0.05% and ethylenediaminetetraaectic acid 0.02% (EDTA). The cells were centrifuged, resuspended and incubated with the graft materials where indicated 4 hours prior to impaction into a pellet under strict aseptic conditions.

Allograft was obtained from fresh frozen femoral heads stored at -80°C. The femoral heads were retrieved in the same fashion as the marrow samples from consenting patients undergoing elective or traumatic hip surgery at Southampton General Hospital in accordance with the local ethics committee. After defrosting the femoral heads they were denuded of soft tissue, osteophyte, cartilage and calcar using an oscillating saw (Stryker Howmedica, UK) and bone nibblers. The remaining bone was coarsely divided and milled using a 3mm bone mill (Aesculap). The smallest morsellised chips were

selected for the study as larger fractions would be difficult to fit into the impactor and perforated cages. The milled graft was repeatedly immersed in 70% ethanol followed by 6% hydrogen peroxide to remove the fat and marrow fractions. Following this the graft was then washed three times in PBS prior to immersion in filter sterilised α–MEM containing Penicillin–Streptomycin (100 mg/500 mL) prior to use. Aliquots of approximately $3cm^3$ of loose graft were placed into separate culture containers prior to seeding.

Seeded and unseeded (control) scaffolds were impacted with a force equivalent to a standard femoral impaction (Brewster, N. T. et al. 1999). The pellet was extruded into the perforated polythene cylinder to keep the grafts in their impacted state (Figure 8). After seeding and impaction the samples were maintained in osteogenic culture media and examined after 7 and 17 day time periods in vitro.

Culture conditions, DNA and alkaline phosphatase-specific activity.

Marrow cells were isolated from 4 patients and cultured under osteogenic conditions, seeded onto allograft at a density of 86,000 cells per sample (n=4) and impacted as shown in Figure 8. Samples were transferred into 12-well tissue culture plates and media added. The plates were incubated at 37°C in 5% CO_2 and media changed every 4 days. Control samples of highly washed morsellised allograft were also cultured under identical conditions. At 1 week half of the constructs were washed with PBS, then incubated with Trypsin / EDTA at 37°C and 5% CO_2 interspersed twice with vigorous vortexing. Cells were collected by centrifugation (13,000rpm for 10 minutes at 4°C), and then resuspended in 1ml 0·05% Triton X-100. Lysis was achieved by freeze – thawing and samples were stored at -20°C until assayed. Lysate was measured for

alkaline phosphatase activity using *p*-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1·5M, pH 10·3 at 25°C (Sigma, Poole, UK). DNA content was measured using PicoGreen according to manufacturer's instructions (Invitrogen, Paisley, UK). Alkaline phosphatase-specific activity was expressed as nanomoles of *p*-nitrophenyl phosphate/hr/ng DNA. This process was repeated at 17 days with the remainder of the samples.

3.3 Impactor design and operation.

Previous force plate analysis studies by Brewster *et al* (Brewster, N. T. et al. 1999) calculated the force of a standard femoral impaction to be equivalent to a 1.98kg mass falling 65mm onto a circular base plate 60mm in diameter. This study necessitated rescaling of the apparatus to produce a small pellet. A 31g weight was dropped 65mm onto an impacting piston 7.5mm in diameter. Calculation of the actual force (i.e. Δ Momentum / Time) imparted on the graft aggregate is problematic due to differing energy absorbing properties of grafts. An "impulse" value was therefore obtained, avoiding the calculation of deceleration time of the impactor onto different densities of graft. This value provides a momentum change, as a given energy, supplied to a unit area of graft aggregate surface:

$$\text{Impulse} = \text{Energy/Unit area} = mgh/\pi r^2$$

$$0.031 \times 9.81 \times 0.065 / \pi (0.00375)^2$$

$$474 \text{ J} / \text{m}^2$$

The impactor (Figure 7) was built by the Department of Medical Physics at Southampton General Hospital. The guiding frame, pellet block, impactor rod and weight were milled from non-corrosive metals to allow autoclave sterilisation. Prior to impaction the apparatus was autoclaved and between samples the parts in contact with tissue were washed with 70% ethanol followed by three PBS washes to avoid sample cross-contamination and cell destruction due to ethanol. Each sample received 72 impactions at a frequency of 1Hz in the manner described by Dunlop (Dunlop, D. G. et al. 2003). After each group of 24 impactions the base was rotated by 120 degrees to prevent adherence of the graft to the base and to effect as uniform an impaction aggregate as possible. The base-piece was rested on sterile gauze to help drain excess fluid forced out of the graft aggregate during the impaction.

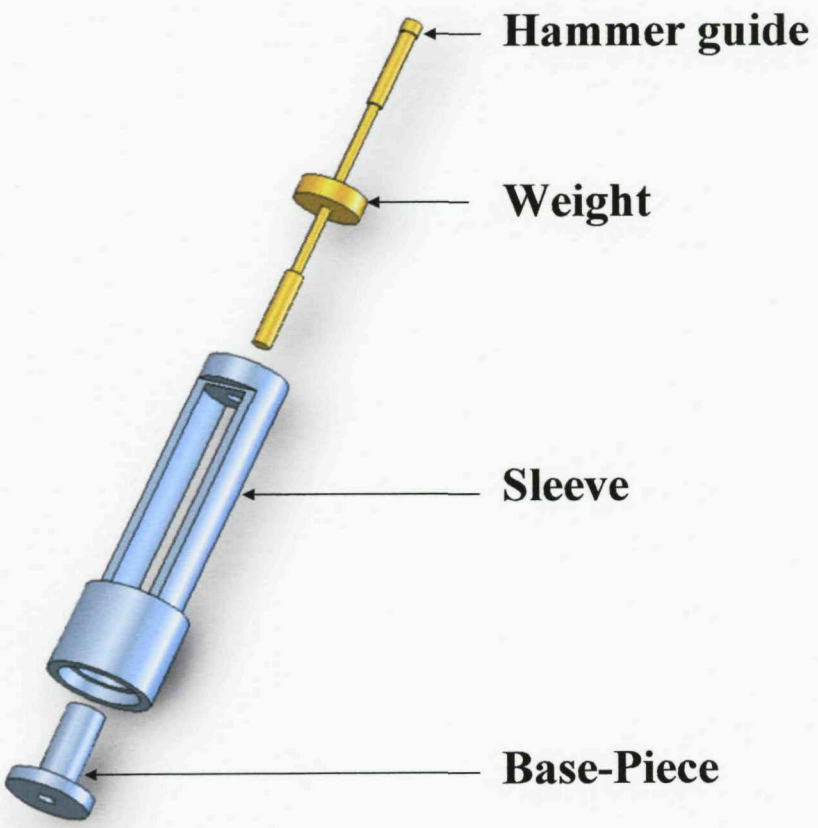


Figure 7. Exploded model of the graft impactor.

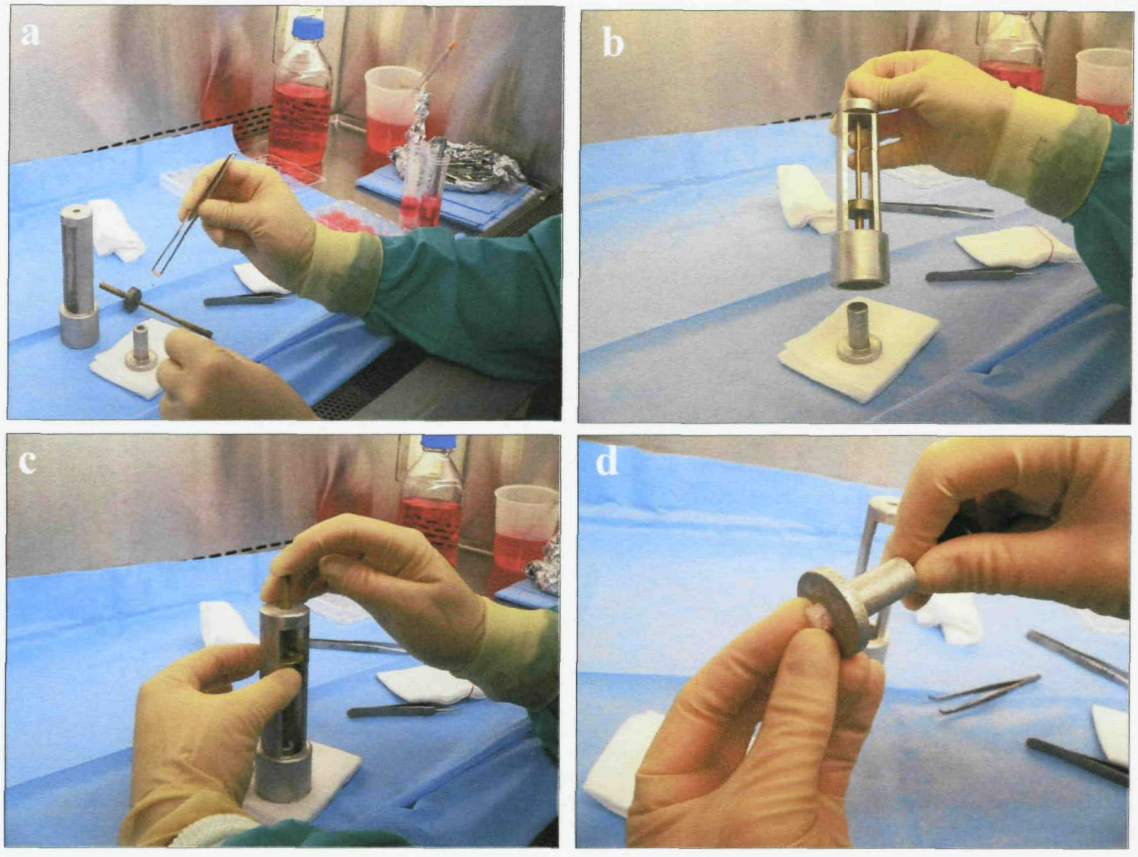


Figure 8. (a) Loading of graft into impactor base-piece. (b) Impactor assembly. (c) Releasing hammer weight. (d) Extrusion of impacted graft into perforated capsule.

3.4 Data analysis

After calibration with lysate controls a best fit curve was obtained to allow calculation of DNA, alkaline phosphatase and therefore specific alkaline phosphatase activity. Sample values were plotted on Microsoft Excel with error bars corresponding to 1 standard deviation (SD) form the mean values.

3.5 Confocal microscopy

Confocal laser microscopy was chosen for this and the subsequent studies for its abilitiy to produce images of thick specimens using a process called optical sectioning. The

ability to section topographically complex specimens such as allograft, TCP and PLA was extremely valuable. All confocal microscopy in this thesis was performed using a Leica TC5 SP2 laser scanning microscope and software.

The process relies on a laser beam passing through a light source aperture which is focussed into a small volume within a fluorescent labelled specimen (Figure 9). For the purposes of the studies in this thesis live cell fluorescent labelling was performed using 5 – Chloromethylfluorescein diacetate (CFMDA "Cell Tracker Green") and dead cell labelling with Ethidium Homodimer-1. The emitted and reflected laser light is gathered by the objective lens, the mixed light is separated by the beam splitter and only the laser light is allowed through and into the detection apparatus. The detected light originating from the scanned volume element represents 1 pixel in the resulting image.

For the purposes of this studythe laser was programmed to scan a 50μm depth using 5 μm slices. This information was used to stack two – dimensional images to generate a three – dimensional image.

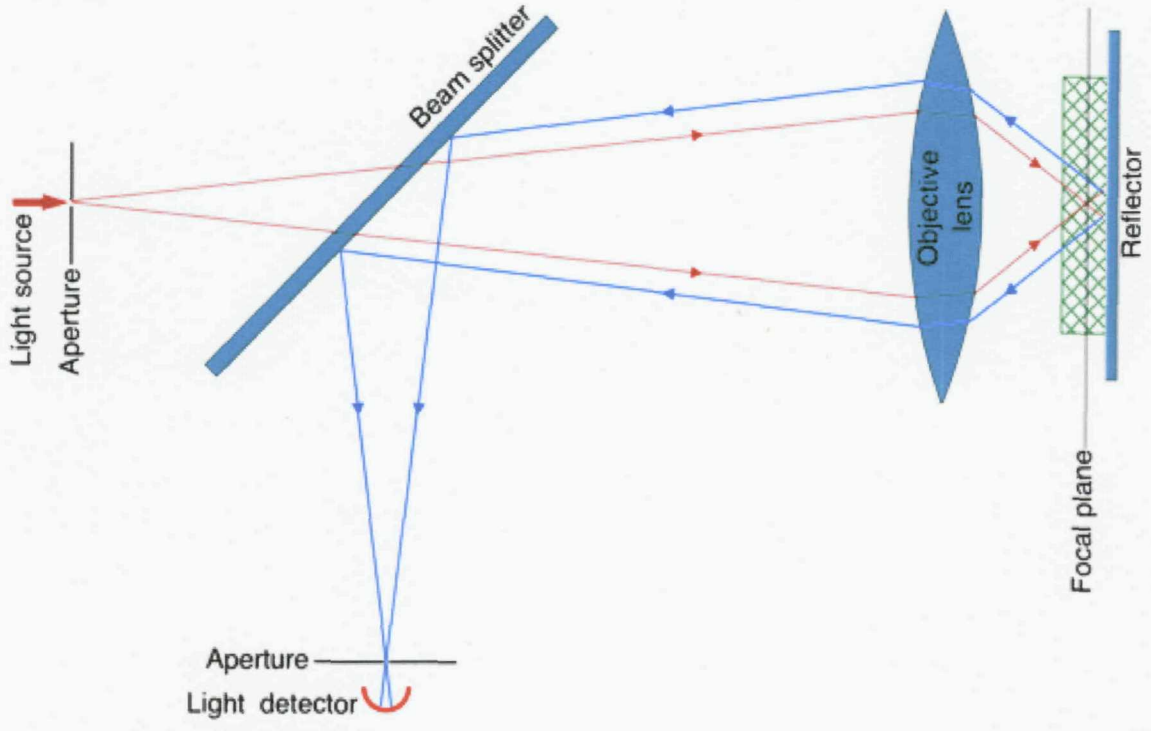


Figure 9. Principles of the Confocal Laser Scanning Microscope. Image courtesy of www.sciencedaily.com.

3.6 Results - Allograft seeded with Human Bone Marrow Stromal Cells

7 and 17 Day in vitro Examination

HBMSC were obtained from 4 patients; 2 male and 2 female with a mean age of 73 (range 63 – 80). Cellular proliferation was observed in all 4 samples as evidenced by increasing DNA content between days 7 and 17 (Figure 10) with 3-4 population doublings over 17 days. Specific alkaline phosphatase activity, reflecting the osteoblast phenotype following culture in osteogenic growth conditions was also confirmed

(Figure 12). Alkaline phosphatase expression increased from 7 to 17 days, although patient-patient variability was observed. The specific activity of the enzyme alkaline phosphatase expressed as Alkaline Phosphatase expression / DNA content, was higher at 17 days compared to day 7 although inter-patient variability was observed as at day 7.

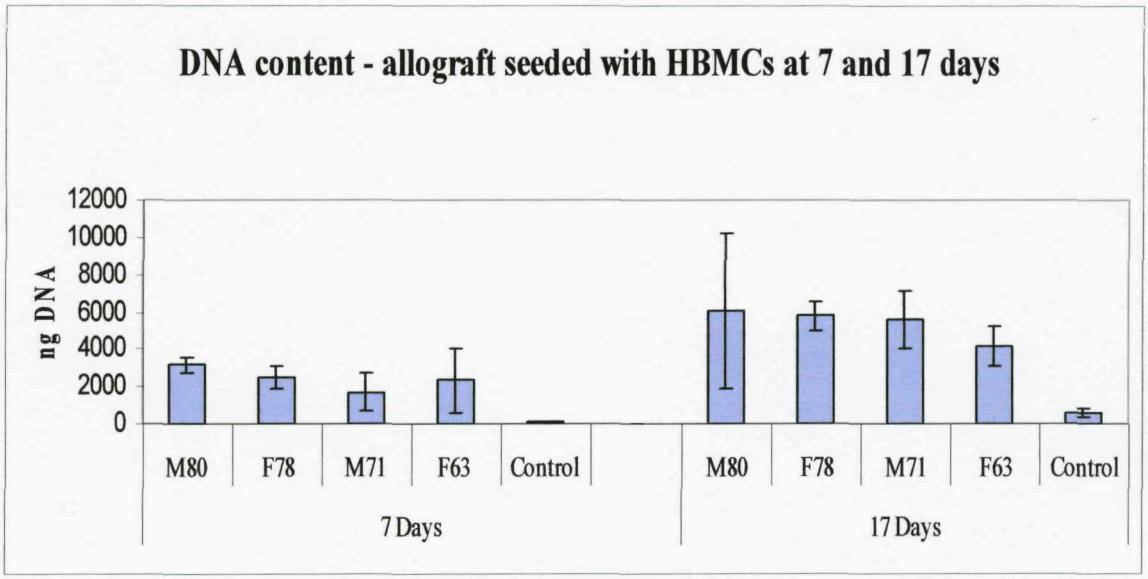


Figure 10. DNA content from the 4 patient samples between day 7 and 17. (Mean +/- SD, n = 4).

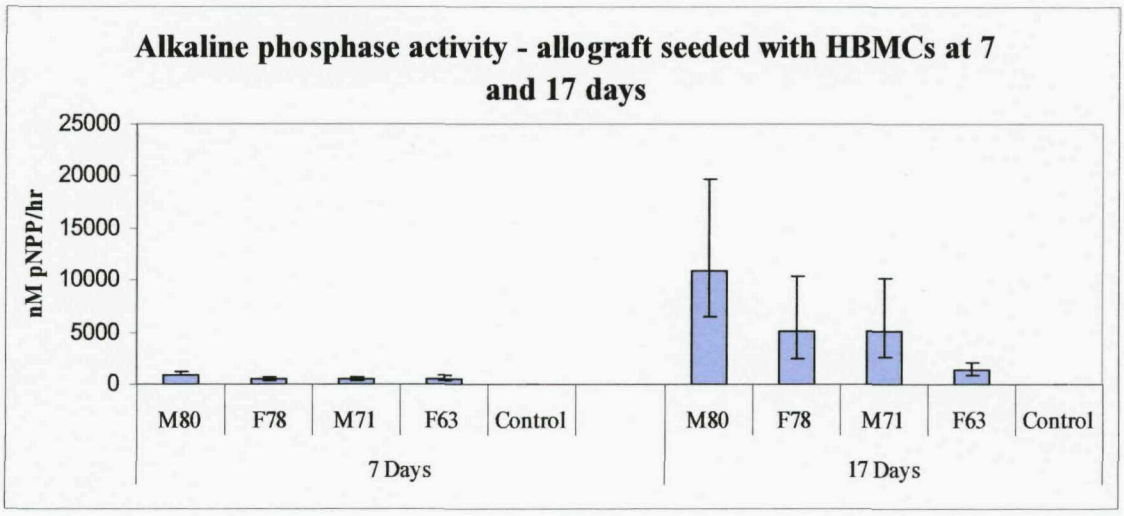


Figure 11. Alkaline phosphatase activity observed from the 4 patient samples between day 7 and 17. (Mean +/- SD, n = 4).

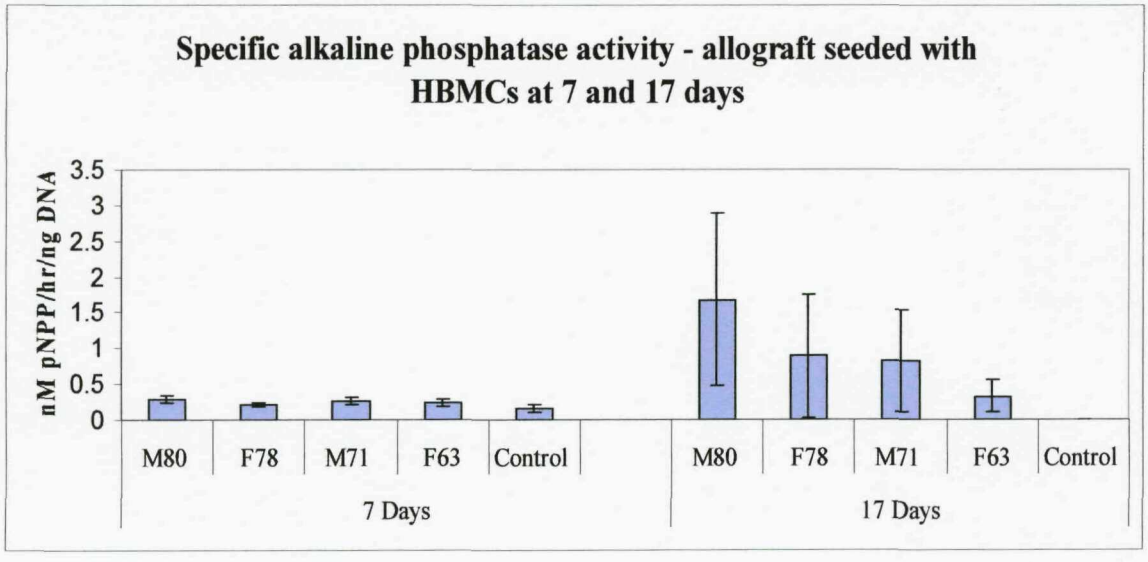


Figure 12. Specific Alkaline Phosphatase activity observed from the 4 patient samples between day 7 and 17. (Mean +/- SD, n = 4).

Live cell fluorescent staining of an aliquot sample from each case confirmed cell viability at 17 days (Figure 13). Confocal microscopy demonstrated live cell proliferation on the allograft surface.

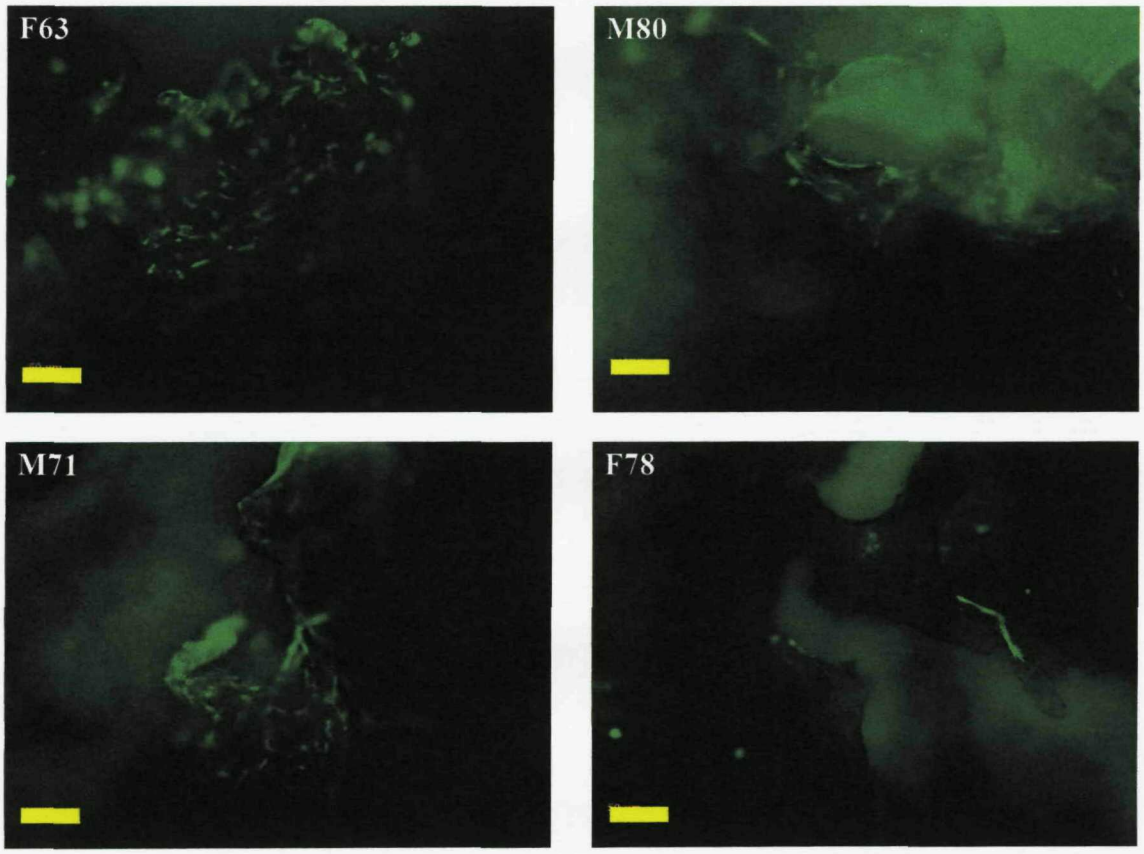


Figure 13. Live cell viability of samples using Cell Tracker Green after 17 days culture; alphanumeric label represents sex and age of marrow sample. Scale bar = 100μm.

3.7 Validation with synthetic grafts

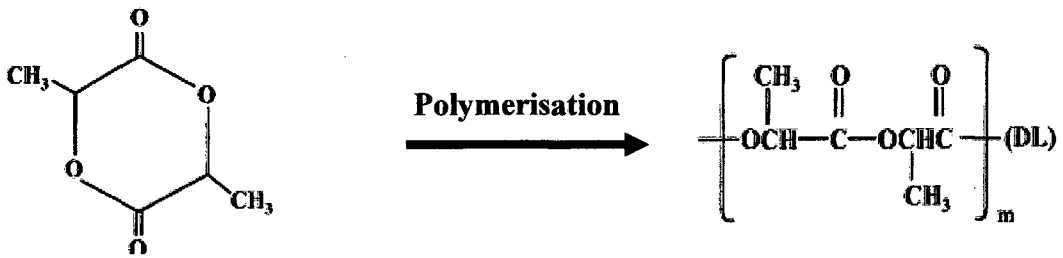
In an identical manner to that described in Study I, the behaviour of HBMSCs on 2 synthetic scaffolds was evaluated. The scaffolds chosen were:

poly(DL-lactic) acid (PLA)

β-TCP (ChronOS™ Synthes Stratec).

3.8 poly(DL-lactic) acid (PLA)

PLA has been used in a wide range of clinical orthopaedic applications, is biocompatible and free of toxicological and immunological hazards. Poly(DL-Lactic) acid is the racemic mixture of the dexo- and levorotary isomers (Figure 14) and is optically inactive.



Lactic acid dimer monomer unit **Poly(DL-Lactic) acid**

Figure 14. Polymerisation of the Lactic acid dimer unit to produce Poly(DL-Lactic) acid. Adapted from Smith & Nephew Endoscopy Division surgeons information sheet.

Degradation occurs predominantly via hydrolysis and in the clinical setting generally takes between 2-5 years to be completely absorbed by the body.

PLA monolith scaffolds produced by Professor Shakesheff's team at the University of Nottingham were used for the study. The texture and morphology of the scaffolds was noted to be similar to that of cancellous allograft.

Production of the PLA monolith scaffolds

0.13g of Poly(DL-lactic acid) (MW 65,000) is mixed in Teflon moulds (10mm deep and 10mm in diameter). The PLA mix was then lyophilised overnight in a freeze drier. The PLA was then foamed using $scCO_2$ to create scaffolds as previously described by (Quirk, R. A. France R. M. Shakesheff K. M. Howdle S. M. 2004;Howdle, S. M. 2001). For this, the polymer was plasticised at 35°C under a pressure of 17.32 MPa. Upon release of the pressure, the pores are formed and fixed in the polymer structure thus creating a porous monolith composite.

Morsellised PLA monolith chips and cancellous allograft were examined at the University of Southampton using an Instron testing rig (Fig 15). Both graft types were initially bathed in α–MEM prior to impaction with an upscaled impactor designed to produce the same impulse of 474 J / m^2 as previously described. The aggregates were cam shear tested under various axial loads designed to keep the grafts in their impacted state. Comparative Stress / Strain curves were determined over a 2.5 mm shearing distance and compared with reference values for solid cortical bone. PLA monolith scaffolds gave a Stress / Strain curve comparable to that of impacted morsellised allograft (Fig 16).

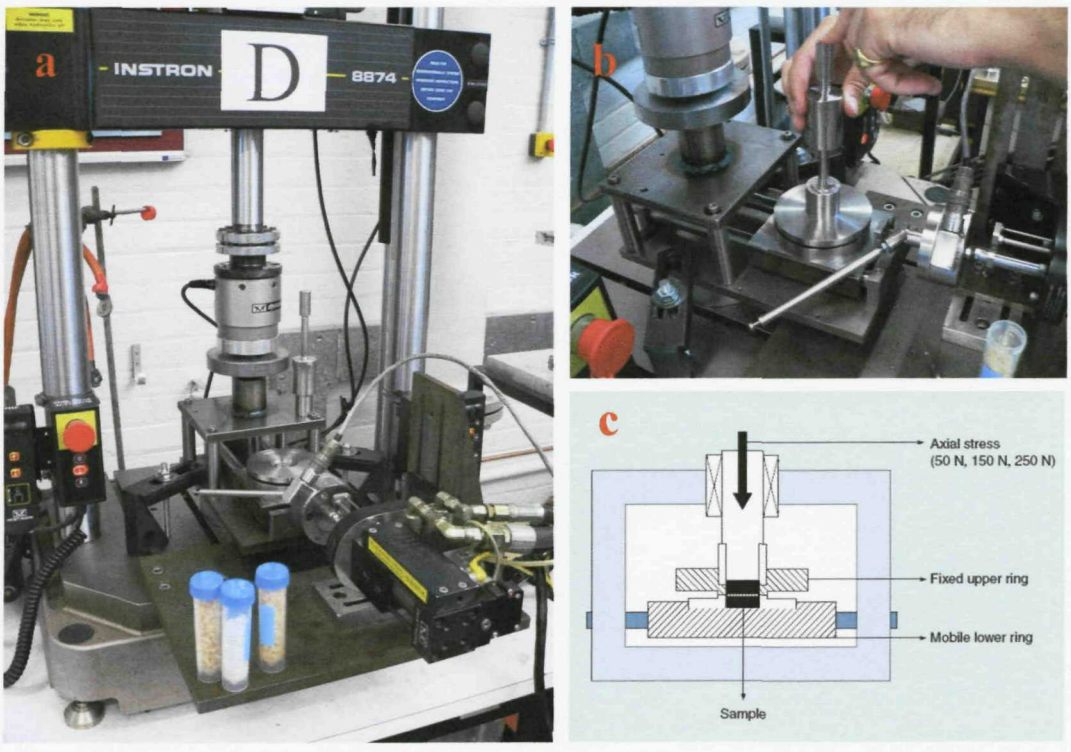


Figure 15. (a) Instron shear tester with samples including PLA chips in centre tube. (b) Impaction of morsellised sample into baseplate. (c) Schematic of the cam shear tester.

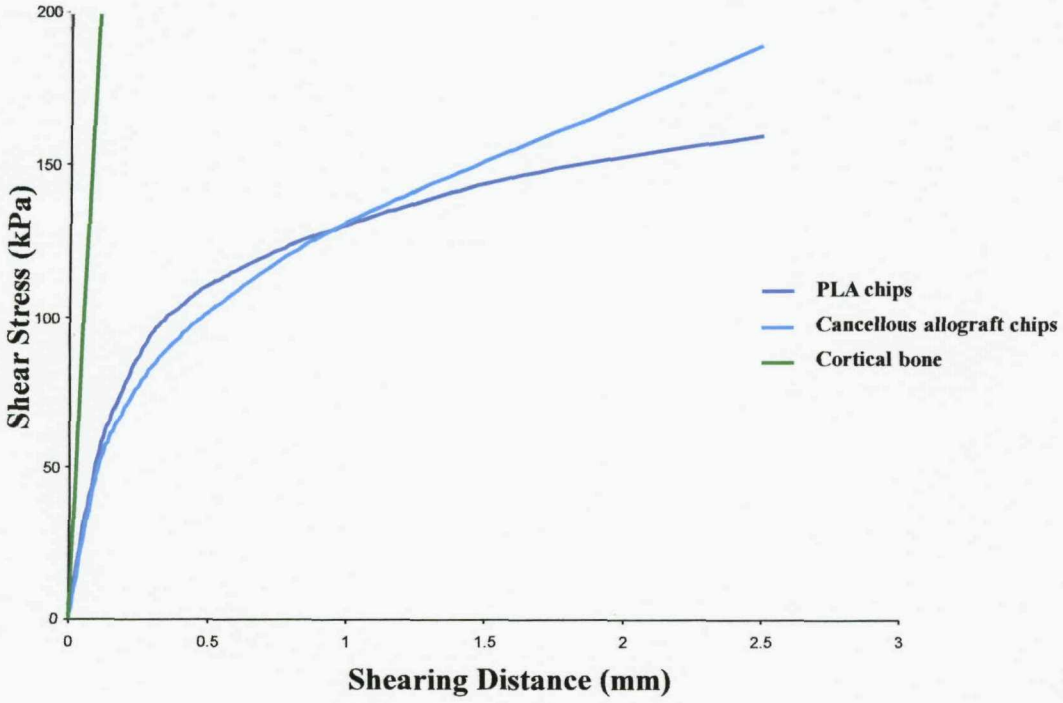


Fig16. Shear testing of impacted PLA chips, impacted morsellised cancellous allograft and solid cortical bone block.

3.9 Method

Grafts from 3 patient donors, average age 76 were seeded at a density of 86,000 cells per sample (n = 4) onto morsellised PLA in an identical manner to that previously described. The PLA was sterilised using the same protocol of ethanol and PBS washes. After incubation the biochemical, histological and biomechanical behaviour was examined at 7 and 17 days.

3.10 Results

Initial graft evaluation would appear to confirm that PLA scaffolds support cell proliferation in vitro.

Biochemical analysis indicated an increasing DNA content between 7 and 17 days. A wide range of values was noted especially in the samples seeded with HBMSCs from the 78 year old female donor (Figure 17). Alkaline phosphatase expression was observed to be considerably heterogeneous, dropping in one patient between the two time points (M80) and undetectable in one patient on initial examination (F78) (Figure 18). The lack of alkaline phosphatase detection in the day 7 run in all 4 samples is likely to have represented a processing or analytical error of the lysate not seen at the 17 day point. This feature skewed the calculation of specific alkaline phosphatase activity in the graft seeded with HBMCs from the 78 year old female donor. Despite identical seeding densities of cells from the same donors there appeared to be a greatly reduced level of recovered DNA and recorded alkaline phosphatase activity when compared to the allograft study.

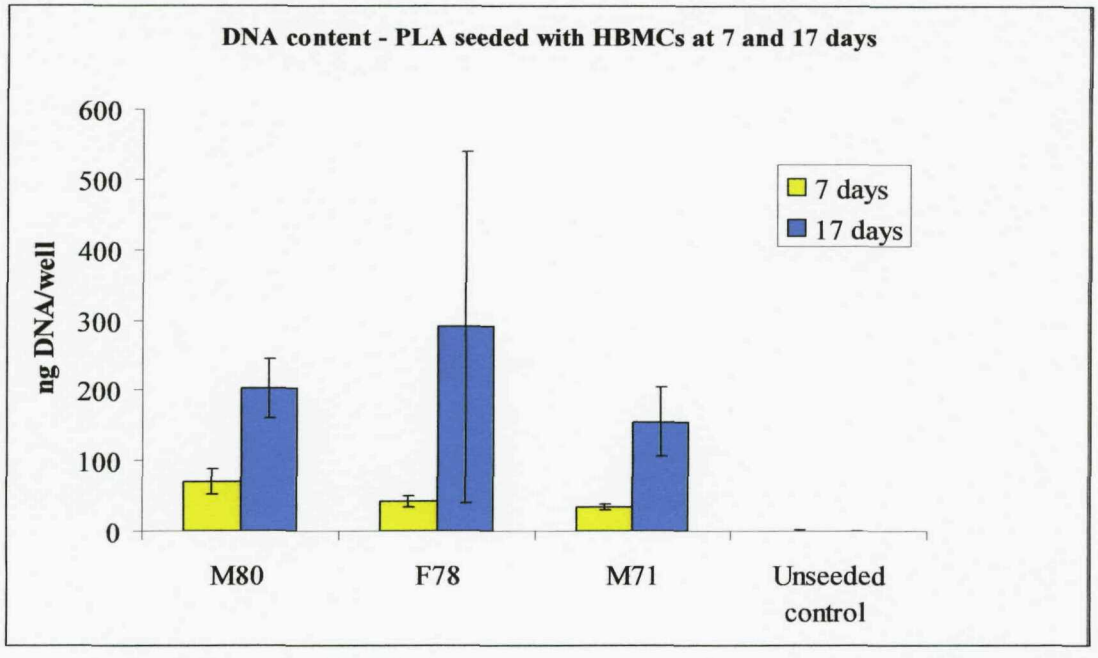


Figure 17. DNA content analysis of HBMCs cultured on PLA scaffold at 7 and 17 days. (Mean +/- SD, n = 4).

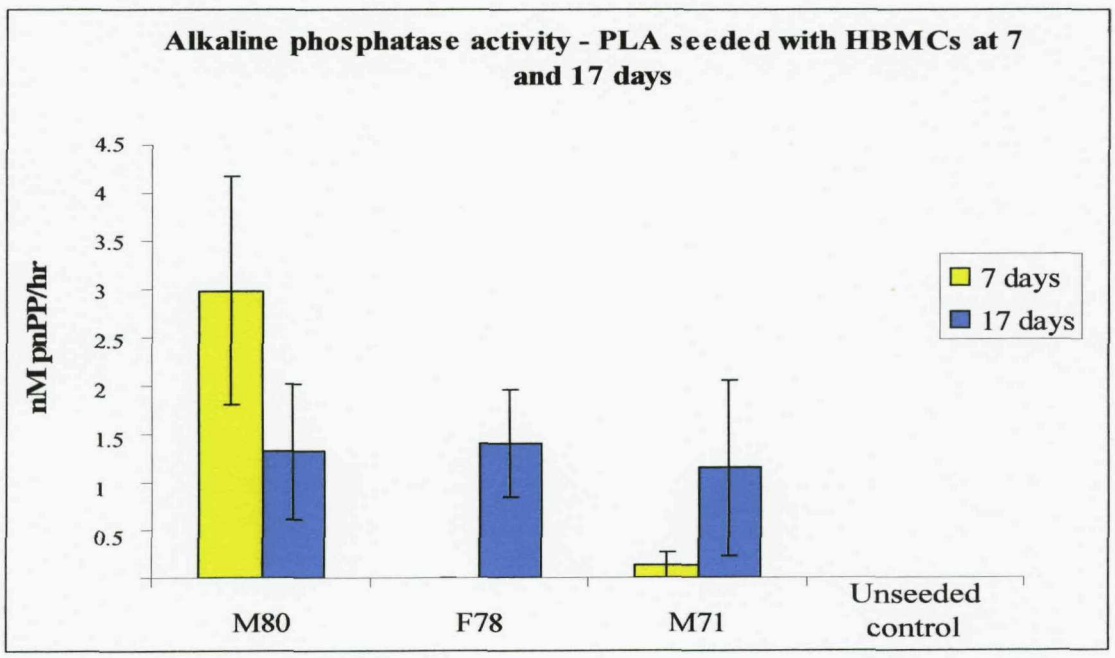


Figure 18. Alkaline Phosphatase Activity analysis of HBMCs cultured on PLA scaffold at 7 and 17 days. (Mean +/- SD, n = 4).

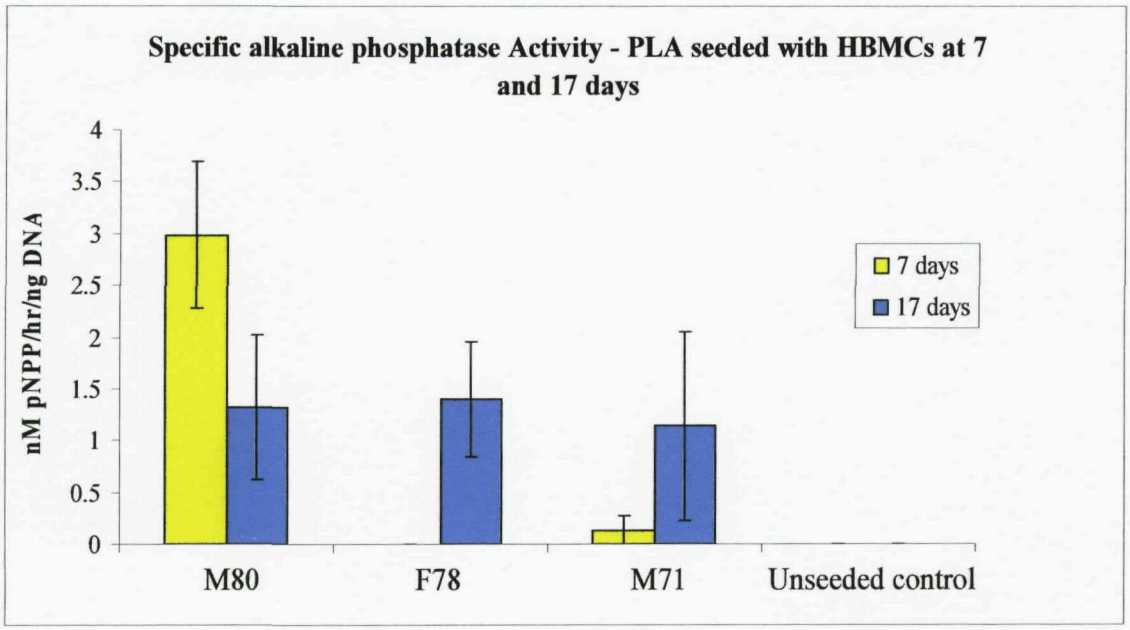


Figure 19. Specific Alkaline Phosphatase Activity analysis of HBMCs cultured on PLA scaffold at 7 and 17 days. (Mean +/- SD, n = 4).

Live cell staining of the cells demonstrated increasing graft population between 7 and 17 days following graft impaction (Figures 20 and 21) with infilling of the graft surface with proliferating cells.

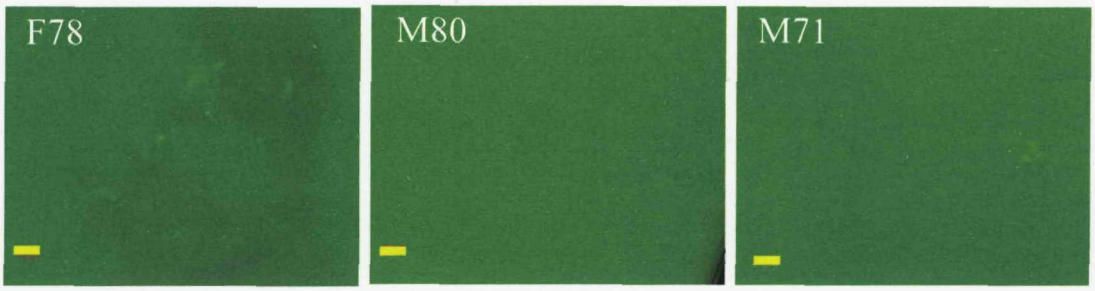


Figure 20. Live cell staining of seeded PLA scaffolds 7 days after impaction.

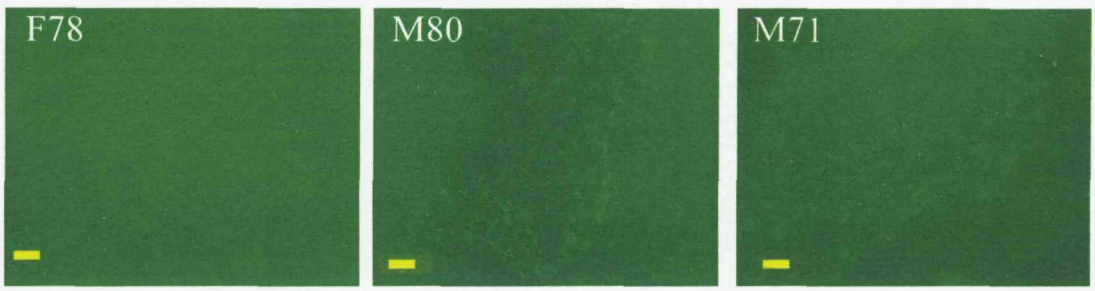


Figure 21. Live cell staining of seeded PLA scaffolds 17 days after impaction.

Alcian Blue and Sirius Red staining was initially used as a screening stain to confirm proteoglycan (Alcian Blue) and more importantly collagen (Sirius Red) production within the samples at 17 days. Further examination using immunohistochemical antibody staining techniques confirmed the collagen to be Type I [Figure 22 (a) and (b)].

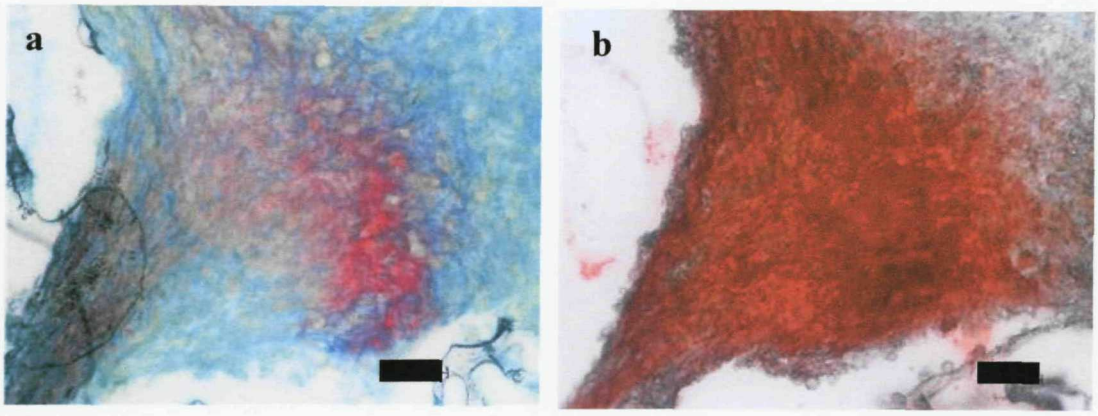


Figure 22. (a) Alcian Blue / Sirius Red and (b) Type I collagen staining of PLA sample following cell seeding, impaction and 17 days culture. Original magnification x20. Scale bar = 50μm. (Images courtesy of Mr B J Bolland).

3.11 β – tricalcium phosphate (β-TCP)

Pure β – tricalcium phosphate [β-$Ca_3(PO_4)_2$] has a long history of successful clinical applications and is widely used in isolation or as a bone graft extender in conjunction with auto- or allograft. No significant adverse reactions have been reported in over 20 years of use (Steffen, T. et al. 2001). The formulation chosen for this study had a compressive strength of 7.5 ±1 MPa, similar to that of cancellous bone which is typically 2 -10 MPa (personal communication Stratec Medical). The material is known to be osteoconductive (Kasten, P. et al. 2003) and is resorbed by the human body within 6 -18 months. Granules measuring between 2.8 and 5.6 mm with a quoted porosity of 60% were used throughout.

3.12 Method

HBMSCs from 3 patients were isolated, expanded, and seeded onto the β-TCP scaffold at a density of 26,000 cells per sample in an identical manner to that previously described. The β-TCP was sterilised using the same protocol of ethanol and PBS washes detailed in the preparation of the allograft. After impaction of the living composite each of the sample groups (n=3) were placed in osteogenic culture media and examined at 24 hours for biochemical behaviour and Live / Dead staining.

3.13 Results

In comparison to the control samples, DNA content (Figure 23), alkaline phosphatase activity (Figure 24) and consequently specific alkaline phosphatase activity (Figure 25) were detected after 24 hours culture. Predictably, DNA and alkaline phosphatase values were lower than in the previous longer term studies reflecting the reduced seeding density and the shorter period of cell culture and associated population doublings.

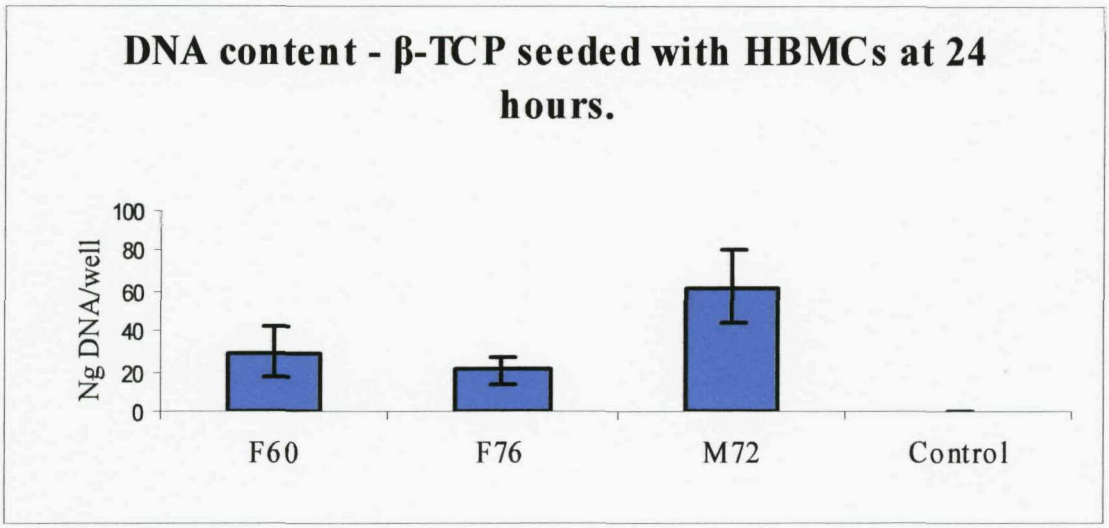


Figure 23. DNA content analysis of HBMCs cultured on β-TCP scaffold at 24 hours. (Mean +/- SD, n=3).

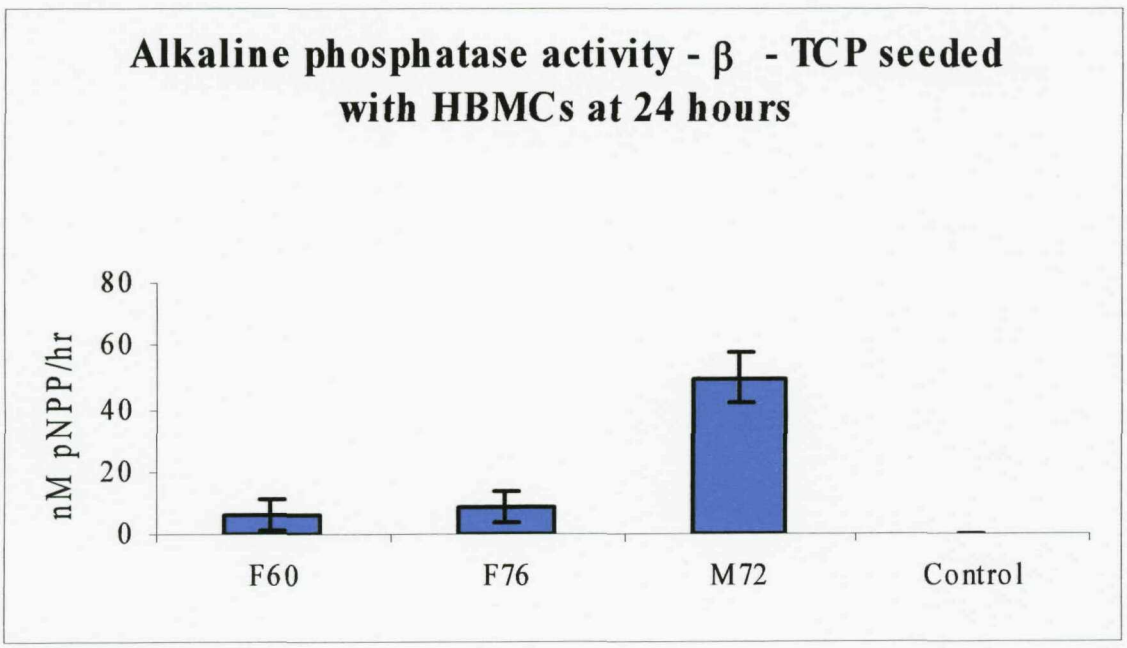


Figure 24. Alkaline Phosphatase activity of HBMCs cultured on β-TCP scaffold at 24 hours. (Mean +/- SD, n=3).

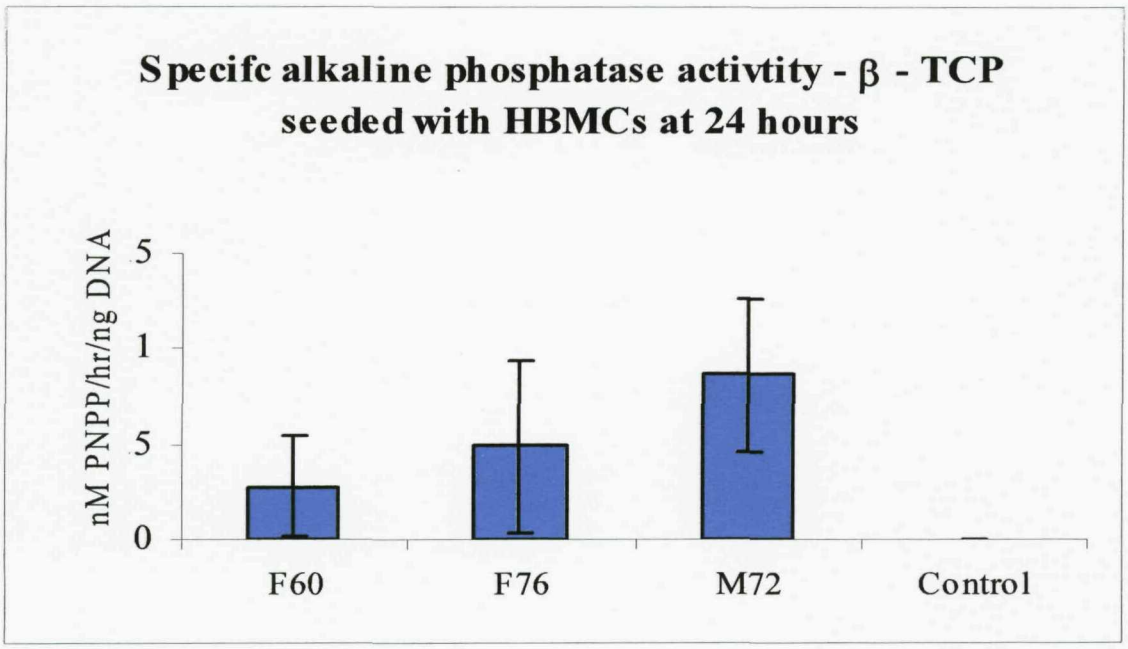


Figure 25. Specific Alkaline Phosphatase activity analysis of HBMCs cultured on β-TCP scaffold at 24 hours. (Mean +/- SD, n=3).

Live cell staining of representative samples of seeded graft (Figure 26) mirrored the Specific Alkaline and Alkaline Phosphatase findings. Graft seeded with cells from the 72 year old male would appear to be more successful than that from the 76 and 60 year old females respectively despite a higher total DNA content from the 60 year old female.

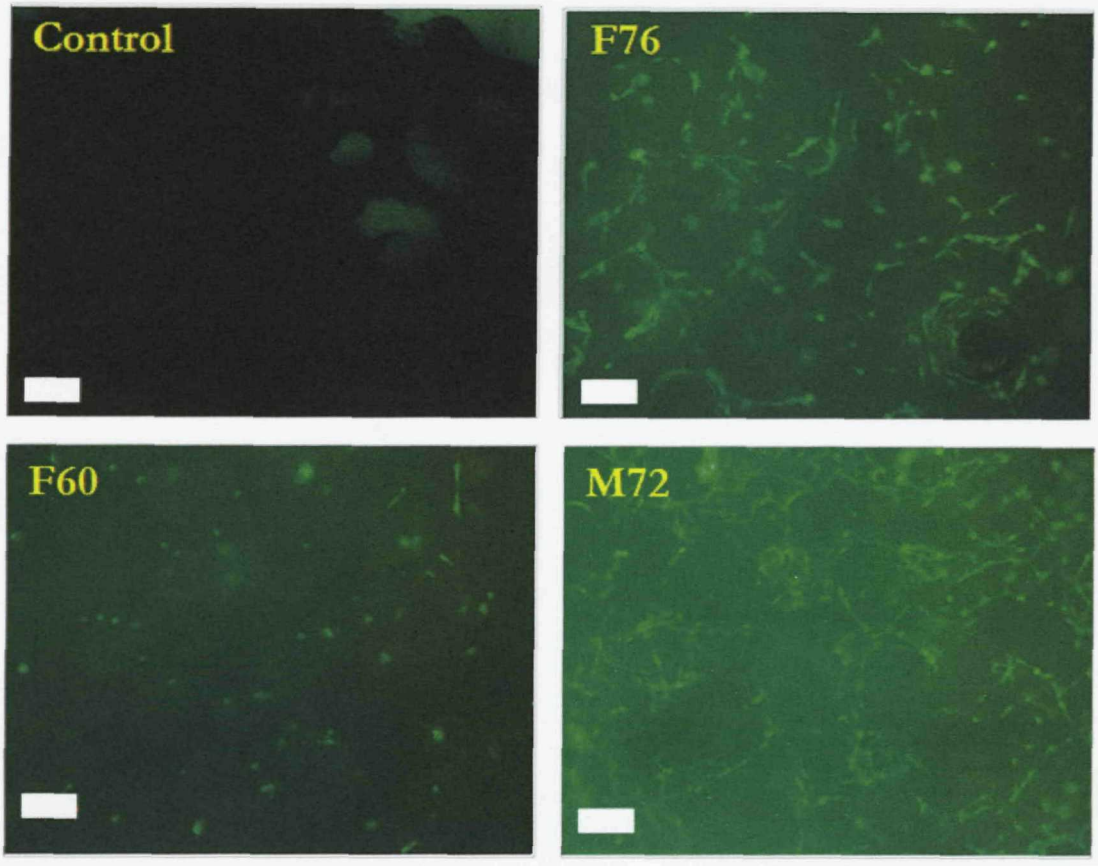


Figure 26. Live cell staining of marrow samples seeded onto β-TCP; alphanumeric label represents sex and age of marrow sample. Scale bar = 200μm.

3.14 Discussion

The potential to encourage bone formation using autologous marrow cells which include mesenchymal populations is an attractive proposition in skeletal regeneration. This study has examined the interaction of impacted human bone marrow stromal cells on washed bone allograft, PLA and β-TCP scaffolds.

The results from this study indicate that seeded HBMSCs can adhere to, survive and proliferate following the physiological process of an impaction procedure in vitro. Large variations in DNA content, Alkaline Phosphatase expression and consequently specific activity, were observed in vitro. The inter-individual variation in DNA content and Alkaline Phosphatase expression at 17 days compared to the day 7 results in the allograft and PLA studies may be partly due to variation in the percentage of osteoblastic cells derived from each donor (Laursen, M. et al. 2003).

Ideally the β-TCP study would have used cells from the same donor, at the same seeding densities with equivalent incubation times as the allograft and PLA studies. This would have allowed a more powerful comparative analysis at the 7 and 17 day time points and a more accurate comparison of the grafts. In reality this was not possible due to contamination of the cells and a reduced seeding volume was achieved with cells from the next batch of 3 patient donors after P1 culture.

Results of the biochemistry would indicate that following seeding and impaction, HBMSCs placed onto allograft more successfully proliferated with an osteogenic phenotype than those added to PLA. Although critically, cells seeded onto the PLA scaffolds displayed evidence of Type I collagen deposition at an early stage.

Standard impaction bone allografting relies on the mechanical properties of dead tissue and although successful in the short to medium term this study set to demonstrate this model could be improved to include the impaction of a living tissue engineered construct. The results are perhaps not surprising using this technique, which represent a

natural evolution of the work originally pioneered by Burwell in 1964 (Burwell, R. G. 1964b).

We were encouraged that the cells proliferated on a non – irradiated bone, this has obvious advantages in the clinical setting particularly with regard to early mechanical stability which is superior in allograft which has not been irradiated.

It was anticipated that the aggressive washing protocol of the allograft reduced the bacterial load (Hirn, M. Y. 2001) and immune response to the graft allowing earlier integration of marrow cells onto the allograft scaffold. In addition to the superior mechanical properties of impacted graft (Dunlop, D. G. et al. 2003) it is anticipated that this technique advance will produce a composite capable of supporting a prosthesis earlier than with standard impaction bone grafting. Indeed, subsequent in vitro mechanical testing has demonstrated that the impacted HBMC/Allograft living composite does provide a biomechanical advantage when compared to allograft alone. Studies by Bolland *et al* (Bolland, B. J. et al. 2006) has demonstrated increased interparticulate cohesion and shear strength when compared to impacted allograft alone. In vivo testing of seeded impacted scaffolds will determine whether these constructs are stronger over longer term time periods with the capability of supporting femoral prostheses at earlier time points. This would have obvious clinical implications with regard to early patient mobilisation and reducing the risk of periprosthetic fracture. If patients could progress from partial to full weight bearing at 2 months rather than 3 the procedure would represent a significant improvement on the current best practice in impaction grafting.

CHAPTER 4

Study II

Parallel in vitro and in vivo analysis of natural (allograft) and synthetic (β-TCP) bone grafts seeded with unselected and immunoselected marrow progenitor cell populations.

4.1 Introduction

This study details the histological findings of a parallel in vitro and in vivo study.in which bone allograft and a β-TCP graft substitute was seeded with unselected or STRO-1^+ selected human mesenchymal stem cell fractions and evaluated in an impaction bone grafting model. Both studies demonstrated cellular viability, activity and osteogenesis after 4 weeks culture in vitro and in vivo.

4.2 Aim

The aim of this study was to evaluate *in–vitro* and the *in–vivo* behaviour of washed morsellised cancellous allograft compared to a β-TCP graft substitute and a 50:50 (by volume) mixture of washed allograft and β-TCP. These scaffolds were seeded with two different mesenchymal stem cell fractions; unselected marrow cells incubated on tissue culture plastic under osteogenic conditions and cells selected as expressing the STRO-1 antigen. This study aimed to mimic the forces that the graft and seeded cells would be subjected to during a human impaction grafting procedure and analysed

their biocompatibility and maintenance of osteogenic behaviour after 1 month, *in–vitro* and *in–vivo*.

4.3 Materials and methods

Reagents, Hardware and Software

Tissue culture reagents were the same as used in Phase I of the study. In addition Ethidium Homodimer-1 was purchased from Molecular Probes, Leiden, Netherlands and collagenase B supplied by Roche, Germany. The β-TCP granules were supplied by Synthes UK. All samples submitted for microtomy were examined undecalcified and were embedded in either glycol (GMA) or methylmethacrylate (MMA).

Isolation and Expansion of Human MSCs

For the purpose of this study, marrow from a 74 year old male who had suffered a fractured neck of femur was recovered as a readily available source and likely to reflect the marrow of a typical impaction bone grafting patient. Marrow preparations of unselected populations and the STRO-1^+ fraction were performed within 24 hours of collection after overnight incubation at 4°C.

Unselected cell fractions were isolated by repeatedly washing the marrow in filter sterilised α–MEM, removing the washed stromal cells prior to centrifugation at 1100rpm and 4°C for 5 minutes. The resultant pellet was resuspended in α-MEM after discarding the fatty layer, filtered with a 70μm filter, counted with a haemocytometer and plated immediately onto T150 flasks. The STRO-1^+ fraction was isolated

according to the method described by Howard *et al* (Howard, D. et al. 2002) using a magnetic activated cell sorting (MACS) system. Both cell fractions were cultured in α–MEM and 10% FCS under osteogenic conditions (50 μg/mL ascorbic acid and 100 nmol/L dexamethasone) repeated at each media change. Penicillin–streptomycin (100 mg/500 mL) was added to the media from the outset. All studies were performed with passage 1 cells (P1). Cells were cultured in flasks at 37°C with 5% CO_2 to confluence and released using trypsin in EDTA. The cells were centrifuged, resuspended and incubated with the graft materials where indicated 12 hours prior to impaction into a pellet under strict aseptic conditions. Scaffolds receiving unselected cells were seeded with 1.4 $x10^6$ cells per sample and those seeded with STRO-1^+ cells received 5.8 x 10^5 cells per sample. The pellet was extruded into the perforated polythene cylinder and maintained in culture media prior to incubation *in–vitro* or *in–vivo*. Loaded scaffolds were impacted with a force equivalent to a standard femoral impaction and contained within a perforated capsule to maintain the grafts in their impacted state. The *in–vitro* group were incubated in osteogenic media while the *in–vivo* arm consisted of identical loaded capsules implanted subcutaneously into 7 SCID mice within two hours of sample impaction. Scaffold and cell allocation for each mouse is summarised in Table 1. Samples from each group were examined histologically after a four week time period. Implantation was performed within 2 hrs of sample impaction and the samples left for 4 weeks.

Mouse	Left flank	Right flank
1	β-TCP Unselected marrow prep	β-TCP STRO-1$^+$ selected cells
2	β-TCP Unselected marrow prep	β-TCP STRO-1$^+$ selected cells
3	Allograft Unselected marrow prep	Allograft STRO-1$^+$ selected cells
4	Allograft Unselected marrow prep	Allograft STRO-1$^+$ selected cells
5	50/50 β-TCP/Allograft Unselected marrow prep	50/50 β-TCP/Allograft STRO-1$^+$ selected cells
6	50/50 β-TCP/Allograft Unselected marrow prep	50/50 β-TCP/Allograft STRO-1$^+$ selected cells
7	β-TCP No seeded cells	Allograft No seeded cells

Table 2. Scaffold and cell type allocation to each mouse.

All of the animals tolerated the in vivo surgical procedure with no obvious inflammatory reactions, infection or cylinder extrusion. The cylinders were easily palpable prior to harvesting and carefully dissected out under sterile conditions. In all samples it was noted that there was a thick vascular membrane firmly adherent to each cage. This membrane was carefully dissected free from the cylinder prior to cylinder extraction (Figure 27). Cylinders were cut into sections with a sterile blade and the sections fixed prior to histological examination. Samples taken from the centre of the pellet were felt to represent the most difficult physiological conditions for the implanted cells to proliferate; these areas were selected for confocal and alkaline phosphatase surface staining analysis. Graft cross sections were also analysed. Confocal microscopy and software was used to capture 50μm image series at 5μm stages using a x20 water immersion lens.

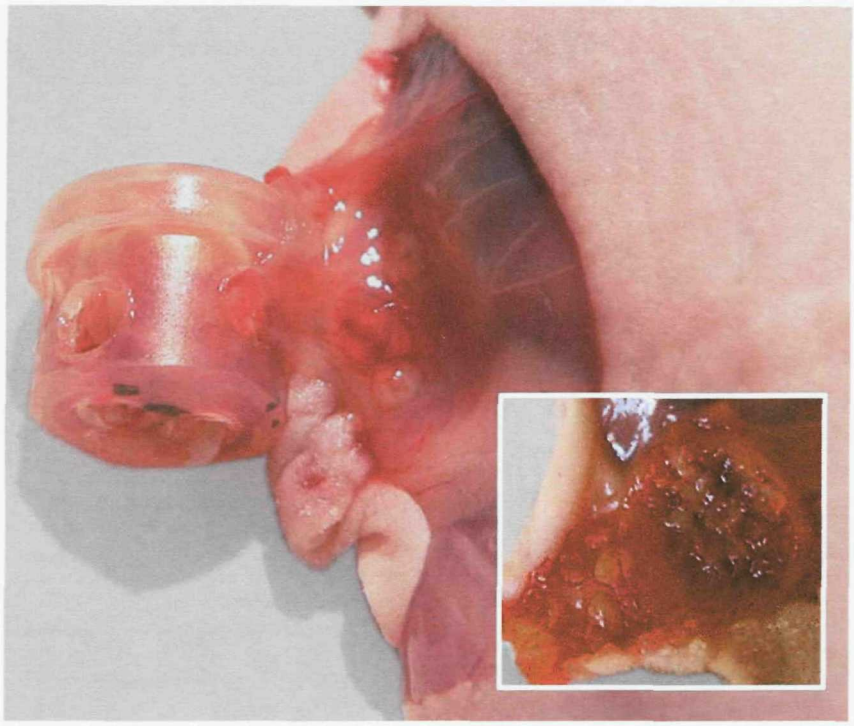


Figure 27. Sacrificed mouse flank showing perforated cage and partially dissected neomembrane. Inset image demonstrating vascular pedicles from the subcutaneous tissue and peritoneal lining. This rapidly established neovascularisation was firmly adherent to the seeded graft.

4.4 Results

In vitro

Confocal microscopy in combination with Cell Tracker Green and Ethidium Homodimer–1 demonstrated viable bone marrow stromal cells in the centre of the pellets seeded with both cell fractions on β-TCP and allograft scaffolds after 1 month incubation. No cells or evidence of cellular activity was seen in the unseeded samples.

Samples from the centre of the bone cages were examined and from this aspect no cell type appeared to be any more successful (Figures 28 and 29). However, when the peripheral populations were examined it was evident both in terms of absolute cell numbers and cell morphology that the STRO-1^+ fraction appeared to populated both scaffolds to a greater extent than the unselected fraction at this time point.

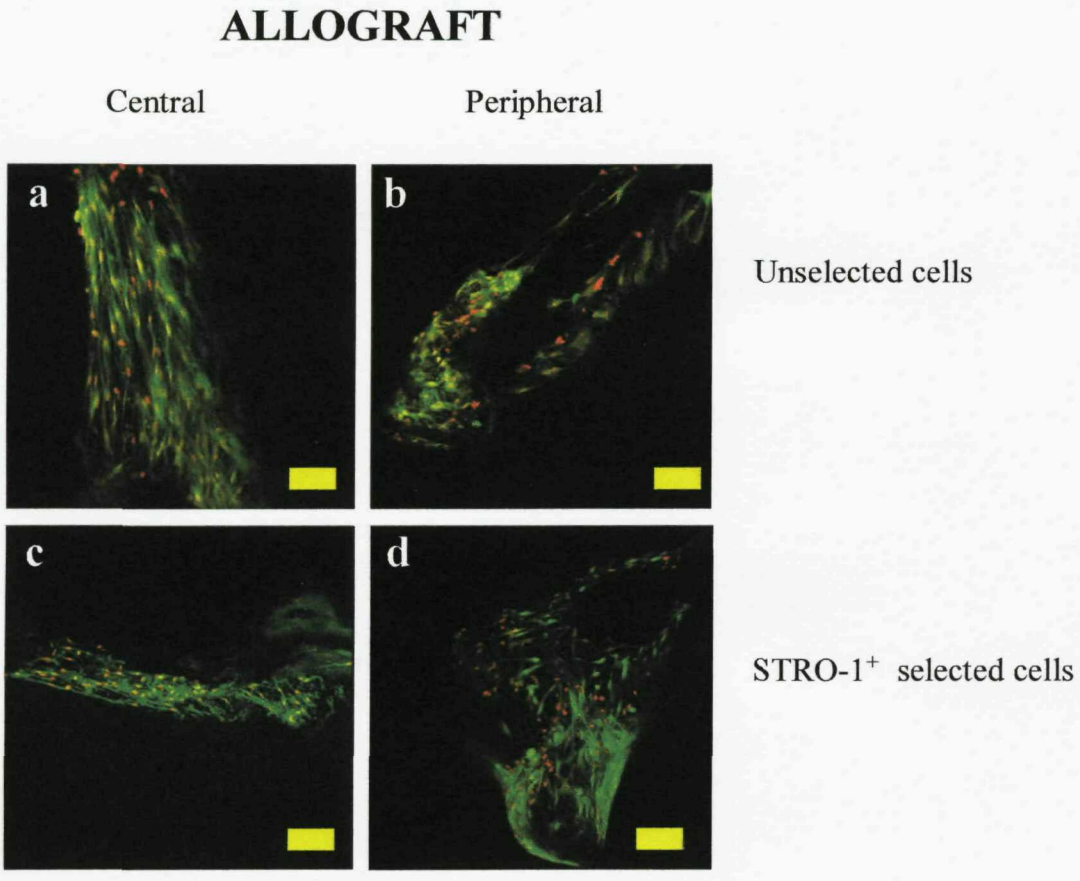


Figure 28. In vitro confocal microscopy images of Allograft scaffold seeded with both cells fractions. (a and c = central and b and d = peripheral samples). Original magnification: x20 scale bar = 100µm.

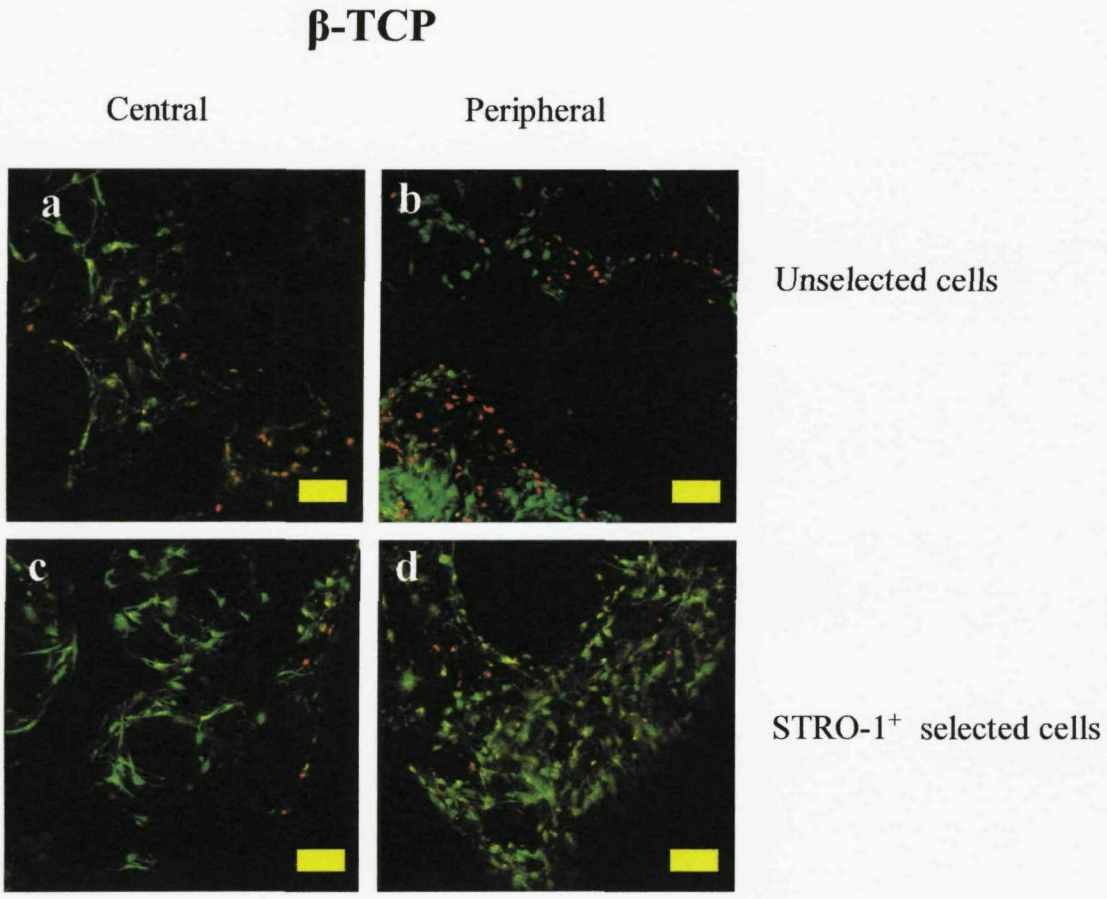


Figure 29. In vitro confocal microscopy images of β-TCP scaffold seeded with both cells fractions. (a and c = central and b and d = peripheral samples). Original magnification: x20 scale bar = 100μm.

Surface staining for alkaline phosphatase expression was positive in all groups seeded with cells. Intense staining was evident with β-TCP/unselected marrow (Figure 30A), allograft/STRO-1^+ cell prep and the 50:50 allograft and β-TCP group seeded with STRO-1^+ selected (Figure 31B) and unselected cells. Lesser staining was observed with allograft/unselected cells.

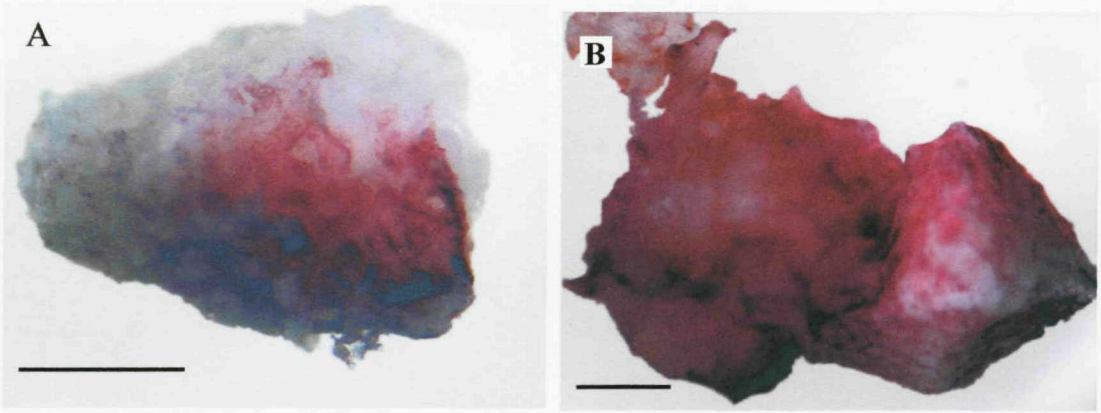


Fig 30. In vitro alkaline phosphatase surface staining of (A) β-TCP seeded with unselected cells and (B) 50:50 β-TCP/Allograft seeded with STRO-1$^+$ selected cells. Scale bars = 1mm.

Toluidine blue staining demonstrated numerous cell populations from both fractions adhering to the surface of the washed allograft. Organised cell populations were observed in the β-TCP/unselected cell group (Figure 31a) and β-TCP/STRO-1$^+$ fraction. In the 50:50 allograft/β-TCP group cells were seen from both fractions populating the graft mixture and bridging the two graft types. Haematoxylin staining of β-TCP samples incubated with STRO-1$^+$ cells demonstrated moderate nuclear staining around the periphery of the sample (Fig 31b).

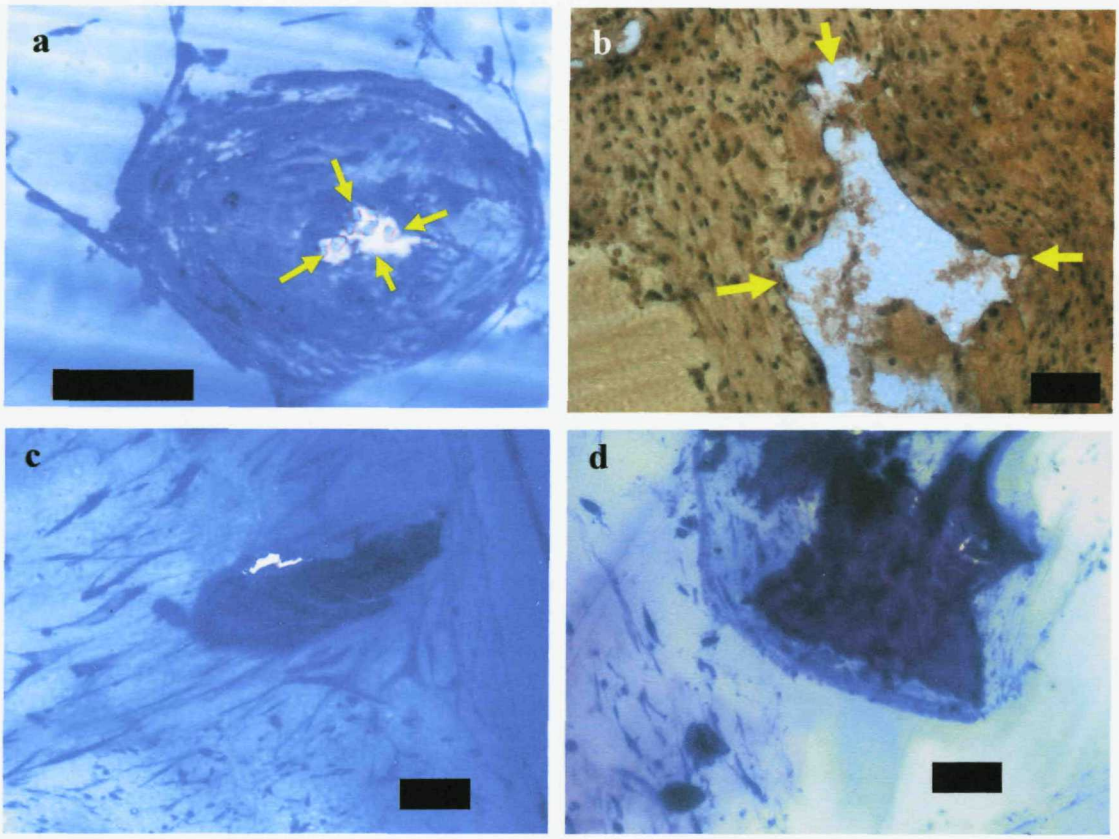


Figure 31. In vitro (a) β-TCP scaffold seeded with unselected cells, Toluidine Blue staining. (b) β-TCP scaffold seeded with STRO-1$^+$ cells, Haematoxylin stain. Arrows outline central core where scaffold was positioned prior to microtomy. The friable nature of the β-TCP proved impossible to obtain full cross sectional images even with resin embedding under high vacuum. (c) and (d) Allograft seeded with unselected and STRO-1$^+$ cells, Toluidine Blue staining. Original magnification (a) = x40, (b-d) = x20. Scale bar = 50μm.

Alcian Blue/Sirius Red staining showed extensive areas of collagen deposition with little evidence of proteoglycan in the β-TCP group seeded with STRO-1$^+$ cells (Figure 32a). Collagen deposition was visualised to a lesser extent in the allograft group. No staining was observed for collagen in the unseeded groups. Goldners Trichrome staining showed enhanced osteoid formation with both scaffolds and both cell

fractions (Figures 32b and 32c). Intense staining was seen on both scaffolds seeded with the STRO-1^+ fraction. Cells were visualised producing an emerging osteoid border over most of the exposed allograft and β-TCP surfaces. No obvious osteoid formation could be detected on the β-TCP graft material seeded with unselected cells as examined using Goldners Trichrome.

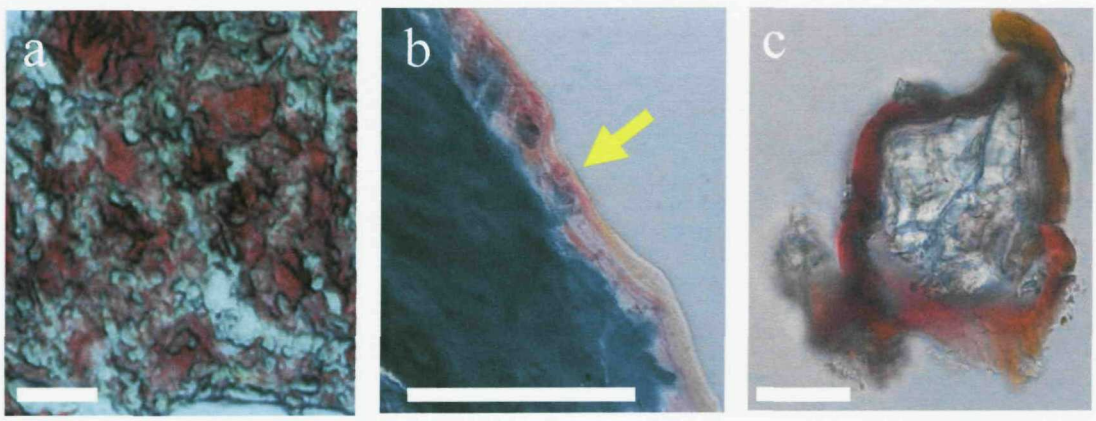


Figure 32. In vitro (a) Alcian Blue / Sirius Red staining of β-TCP plus STRO-1^+ selected cells. Goldners Trichrome staining of (b) Allograft seeded with unselected cells (arrow denotes emerging osteoid border) and (c) β-TCP seeded with STRO-1^+. Green = mineralised allograft, orange/red = osteoid, and blue/grey = nuclei. Original magnification (a) = x20, (b) = x40, (c) = x100 (scale bar (a and b) = 50μm, (c) = 10μm.

In vivo

Primary human bone marrow and immunoselected human bone marrow cells were impregnated onto allograft and β-TCP graft, impacted and implanted into nude mice as described in the materials and methods. After four weeks, chambers were removed and analysed for cell viability, activity and new osteoid formation (see Table 2 below).

	In vitro				In vivo			
	Confocal (Live/ Dead)	Alkaline Phosphatase	Alcian Blue/ Sirius Red	Goldners	Confocal (Live/ Dead)	Alkaline Phosphatase	Alcian Blue/ Sirius Red	Goldners
Allograft Alone	-	-	-	-	-	-	-	+
Allograft + Unselected Cells	+++	+	+	++	+	-	+	+++
Allograft + STRO-1+ cells	+++	+	+	+++	++	-	+	+++
ß-TCP alone	-	-	-	-	-	-	-	-
ß-TCP+ Unselected Cells	+++	+++	+++	+	++	+	++	+
ß-TCP + STRO-1+ cells	+++	++	+++	+++	+++	+	++	+

Table 3. Visual Analogue Scoring of live and dead cell numbers/deposition using laser confocal microscopy, degree of alkaline phosphatase staining, collagen deposition (Sirius Red) and osteoid production (Goldners Trichrome). All groups graded as: - Absent, + Mild, ++ Moderate, +++Heavy.

As observed in the in vitro studies, confocal microscopy demonstrated live cells at the centre of all pellets seeded with both cell fractions. The proportion of live cells was reduced in comparison to the in vitro model but cell numbers were qualitatively higher than the unseeded pellets which had negligible live cell activity at 1 month (Figure 33 e, f, k and l). In addition the morphology, especially of the β-TCP sample seeded with STRO-1^+ selected cells (Figure 33 c and d) was clearly different from the unseeded cells. The appearances were similar to the in vitro group with abundant cells and large amounts of matrix adhering to and outlining the macropores. As with the in vitro group enhanced live cell numbers were seen at the periphery of the pellets when compared to central samples. This may reflect a shorter nutrient diffusion distance and/or the establishment of new blood vessels around and into the cage (Figure 27). In all cell / graft constructs blood vessel encroachment and travel through the cage perforations could be observed with robust attachments to the periphery of the graft aggregate.

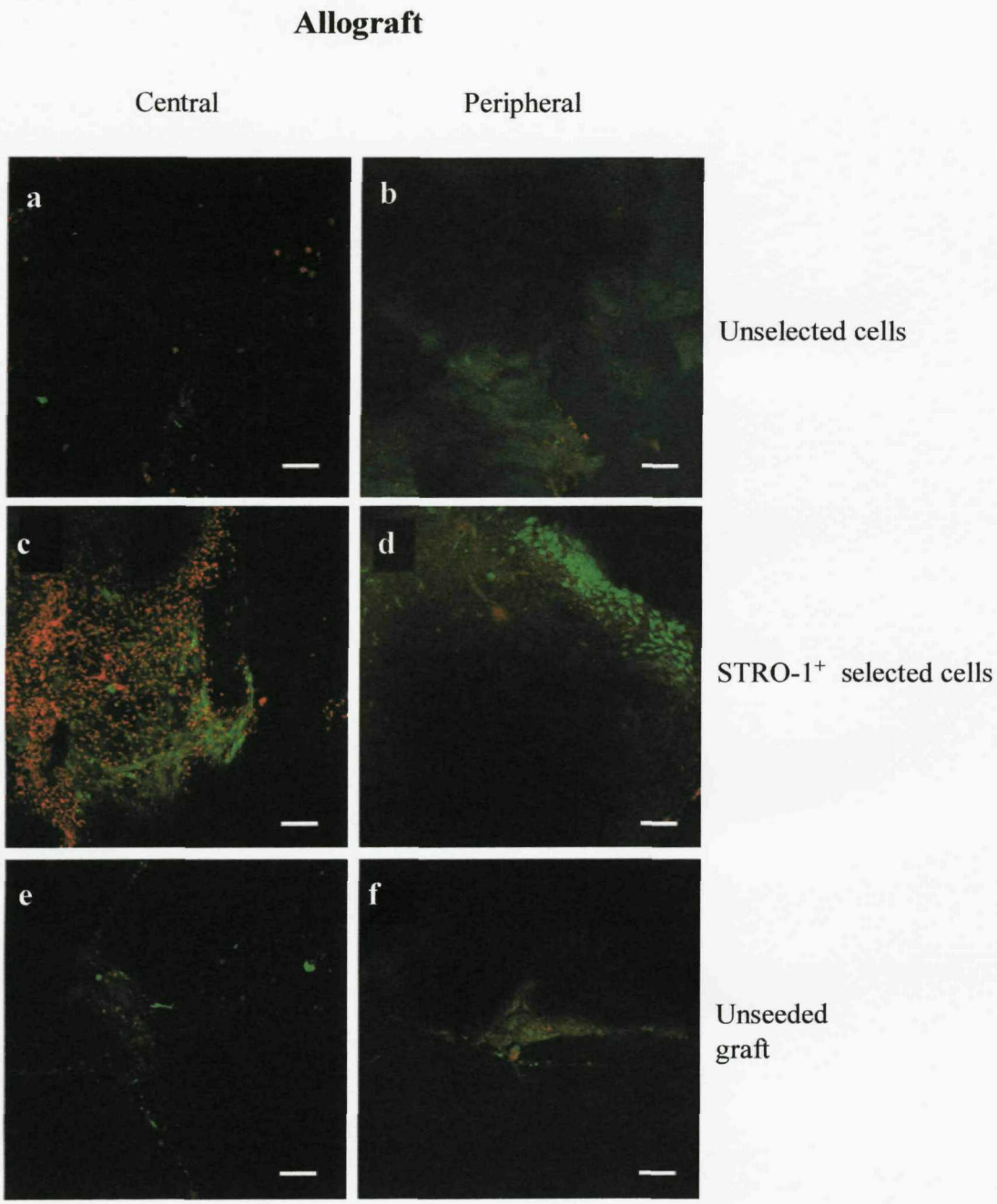


Figure 33. In vivo confocal Microscopy of seeded and unseeded Allograft using Cell Tracker Green and Ethidium Homodimer-1 (Live / Dead) staining. Scale bar = 80μm.

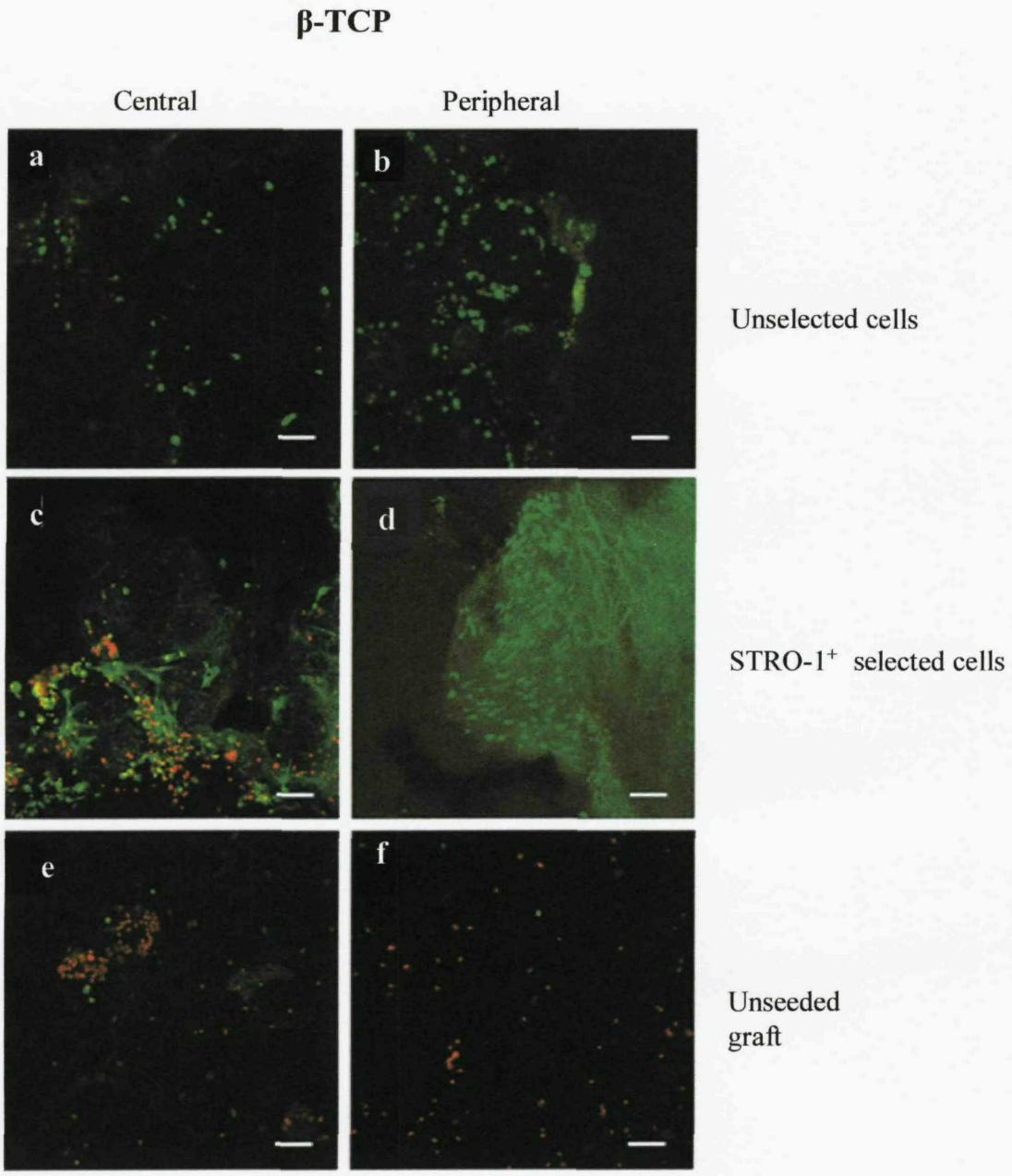


Figure 34. In vivo confocal Microscopy of seeded and unseeded β-TCP using Cell Tracker Green and Ethidium Homodimer-1 (Live / Dead) staining. All images at original magnification of x20. Scale bar = 80μm.

Cell matrix and new osteoid formation was determined using Toludine Blue, Alcian Blue/Sirius Red and Goldners Trichrome staining. As observed with the in vitro samples, good cell viability, adherence and proliferation was observed in cell/scaffold constructs in the in vivo group. The seeded samples expressed qualitatively more collagen and osteoid (Figure 35) in comparison to the unseeded groups. β-TCP appeared to act as a better substrate for new collagen deposition when compared to allograft with both cell fractions, but a higher amount of osteoid production was seen using Goldners in the allograft group seeded with both cell types.

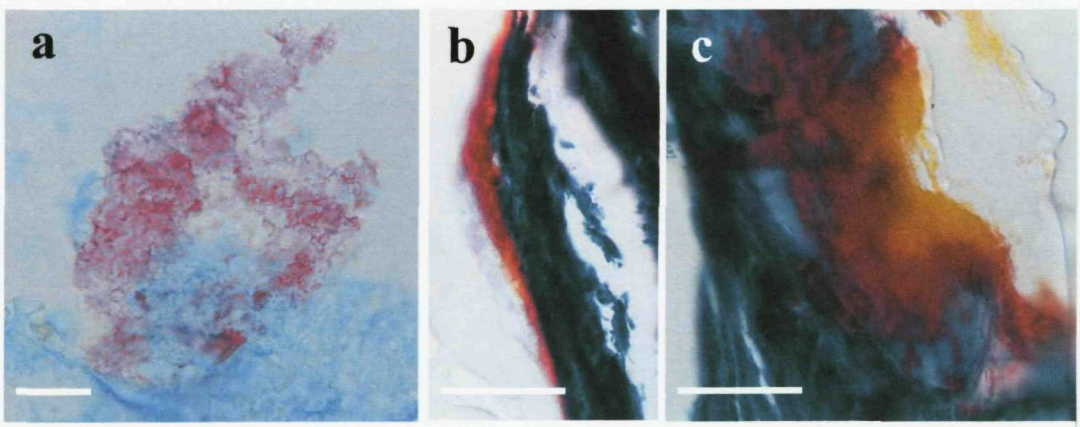


Figure 35: In vivo. (a) β-TCP seeded with unselected cells. Alcian Blue/Sirius Red staining demonstrating collagen matrix deposition. (b) Allograft seeded with unselected cells. Goldners Trichrome. (c) Allograft seeded with STRO-1^{+} cells. Goldners Trichrome. Original magnification (a) = x100, (b) and (c) = x40. Scale bar: (a) = 10μm, (b) and (c) = 50μm.

Surface staining for alkaline phosphatase was almost universally absent in all samples. Small isolated areas were detected on the β-TCP samples seeded with both MSC fractions but negligible activity was seen elsewhere.

4.5 Discussion

This study has examined the interaction of impacted seeded MSC fractions on washed bone allograft with and without the addition of ß – TCP graft substitute. The current results indicate the viability of unselected and selected human bone marrow stromal populations following impaction and their potential to augment bone formation. The results would indicate that the STRO-1^+ selected cells more successfully populated the scaffolds and would therefore be the cell fraction of choice in the clinical setting.

The in vitro and in vivo results suggest that β-TCP graft and allograft are viable scaffolds on which to seed HBMSCs and osteoprogenitors (STRO-1^+ selected) prior to impaction bone grafting. Indeed the morphology, live cell numbers, collagen and osteoid production demonstrated in the previous figures and summarised in Table 2 would indicate that β-TCP graft is possibly more successful than the currently accepted washed morsellised allograft.

Home office restraints on the number of small animals to be used meant that different graft and cell types were implanted into the flanks of the same mouse. While the subcutaneous cylinders were anatomically separated there remains a possibility that the effects observed may be due to a systemic humoral response. Future studies would ideally utilise single specimens in one flank only.

It is important to note that the seeding volume of the immunoselected cells was approximately half that of the unselected cells after processing (5.8×10^5 compared to 1.4×10^6 cells per sample). Ideally the seeding volumes would have been identical but it was felt important to examine the numbers with and without immunoselection after

P1 culture as these would be the expected numbers seen in the clinical setting after approximately 14 days of ex-vivo expansion.

This initial enthusiasm should be tempered for a number of reasons; namely the long and complex processing time when compared to isolating unselected fractions (eight hours compared to one hour) and the markedly donor dependent expression of STRO-1 (Stewart, K. et al. 2003). In addition the higher cell division rates seen in STRO-1^+ selected as opposed to the unselected fractions (MacArthur B, Oreffo R O C; unpublished data) may actually be detrimental to the graft in the short term by encouraging early graft resorption, potentially destabilising any prosthesis that it was supporting. A similar phenomenon was encountered with the use of OP–1 (BMP–7) in a femoral impaction grafting sheep model (McGee, M. A. et al. 2004).

Cellular viability to withstand the forces produced in a standard femoral impaction both in vitro and in vivo has been demonstrated with both cell fractions after 4 weeks incubation. Critically, these cells continued to respond to osteogenic stimuli or were already committed to produce a bony matrix at this point.

CHAPTER 5

Study III

Proof of concept clinical translation of methods from laboratory to operating theatre.

5.1 Introduction

This study details laboratory and clinical findings from two clinical cases, where different proximal femoral conditions (a benign proximal femoral tumour and avascular necrosis) were treated using impacted allograft augmented with marrow-derived autogenous progenitor cells.

Both patients were appropriately counselled preoperatively and understood that the proposed treatment was a combination of accepted clinical procedures and as such ethical approval was not sought.The components of the technique included drilling / decompression to the area of pathology and retrograde impaction of highly washed allograft seeded with bone marrow aspirate.

CASE 1

5.2 Pathophysiology of benign proximal femoral tumours.

Benign tumours are commonly found in the proximal femur (Jaffe, K. A. et al. 1990). Clinical presentation is variable depending on the pathology, site and size of the lesion. The spectrum of presentation varies from asymptomatic incidental x-ray

finding to those with acute proximal femoral fracture. Most however present with pain, limp and leg-length inequality (Shih, H. N. et al. 1996).

Nonoperative observation is the first choice of treatment but occasionally the degree of bony destruction renders the area at risk of a pathological fracture. Qualitatively, indications for surgery include progressive lesions causing pain or deformity and quantitatively if over 2·5 cm or involving at least 50% of the cortex where there is a significant risk of fracture (Mirels, H. 1989;Jaffe, K. A. et al. 2002).

Principles of prophylactic treatment remain contentious and may involve curettage of the lesion, void filling either with bone graft or marrow injection and mechanical stabilisation. The process of reconstitution of the defect is often difficult due to the degree of bone loss. In addition, reconstruction may be associated with significant blood loss, donor site morbidity, infection, restriction of activity and recurrence (Roposch, A. et al. 2004).

5.3 Case History

A sixty two year old male presented with a one year history of right hip pain. The patient described a dull ache around the lateral trochanteric region, passing down the anterior thigh with significant deterioration in the months leading up to the procedure. The patient reported difficulty in walking and was beginning to experience night pain, although otherwise the patient was systemically well with no significant past medical history. On examination he walked with an antalgic gait. There were no other positive findings and no restriction of hip movements.

Plain radiographic (Figure 36a and b) and Magnetic Resonance Imaging showed degenerative changes in both hips, more advanced on the opposite (left) side. A well circumscribed focal lesion was seen in the anterior portion of the femoral neck on the right side. The lesion was expansile with slight distortion of the anterior femoral neck cortex (though this remained intact) and diagnosed as representing a benign non-ossifying fibroma.

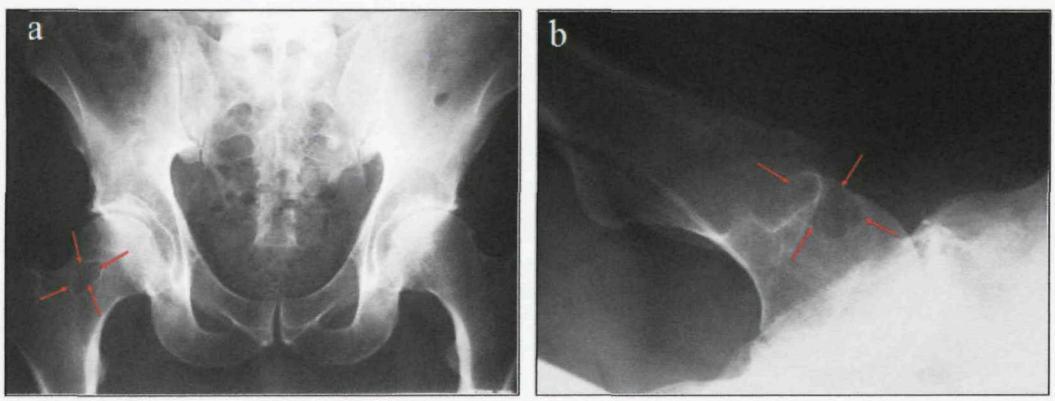


Figure 36. Pre-operative Anterior-Posterior (a) and Lateral (b) pelvic and right hip radiographs of Case 1. Red arrows outline the margins of the lesion.

Exacerbation of the patient's pain and proximity of the lesion to the anterior cortex indicated the potential of impending fracture through the lesion. Surgical intervention was recommended with appropriate counselling including the risks of intra-operative femoral neck fracture.

5.4 Surgical Technique

Morsellised allograft was prepared from a banked fresh frozen femoral head. After denuding the femoral head of articular cartilage, fibrous tissue, osteophyte, cystic

areas and calcar using an oscillating saw (Stryker UK) the remaining bone was milled using the large and small cylinders of a Noviomagus bone mill. This combination of mill sizes ensured optimal particle size distribution (grading) most resistant to shear strength (Dunlop, D. G. et al. 2003). The morsellised aggregate was serially washed with pulsed lavage and 6% Hydrogen Peroxide until no further fat or blood was visible. The graft was then finally washed with normal saline and mixed with 500mg Vancomycin powder.

Bone marrow aspiration was performed with the patient in the lateral position in the operating theatre under strict aseptic conditions. A single incision was made over the posterior superior iliac spine and multiple passes were made with a trephine to prevent dilution of the aspirate with haemopoietic cells (Figure 37). A total aspirate volume of 36 mls was obtained, 2mls were retained for ex-vivo analysis and the remainder immediately seeded onto the graft and covered.

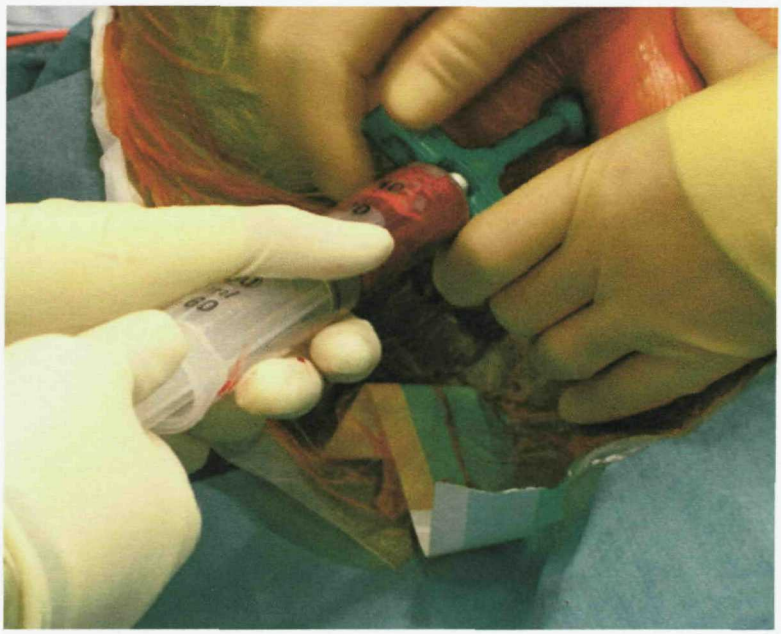


Figure 37. Aspiration of bone marrow from the posterior iliac spine.

The patient was then positioned supine on the operating table with standard lateral preparation and draping. Image intensification was used to determine the incision point. A 1·5 cm incision was made in the skin over the lateral thigh followed by sharp dissection to bone. A guide Kirschner wire was introduced through the lateral cortex and under image intensification directed towards and through the cystic lesion. A 10mm cannulated femoral canal corer (Stryker Int. IBG kit) was passed over the Kirschner wire (Figure. 38b), removing a tunnel which included the contents of the cyst, which was used for histological analysis. Aggressive curettage using a serrated spoon was performed to the cyst walls under image intensification.

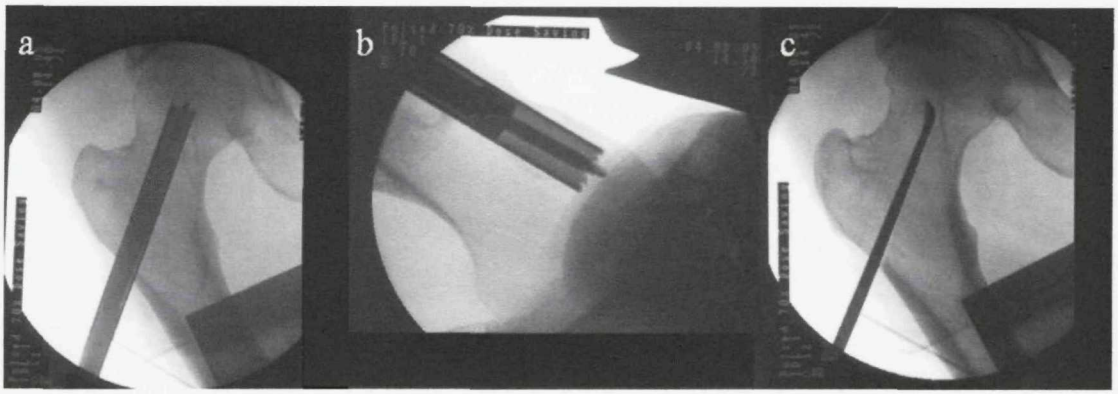


Figure 38. Anterior-Posterior (a) and Lateral (b) intra-operative images of the apple corer being railroaded over the K-wire into the cyst cavity. Curettage of the cavity walls (c).

Graft was introduced into the corer (Figure 39a) and impacted ex-vivo to produce a solid core. This was extruded with further impaction in a retrograde fashion into the tunnel defect under image intensified control. This process was repeated with grafting to the lateral femoral cortex. The wound was closed following haemostasis and washout.

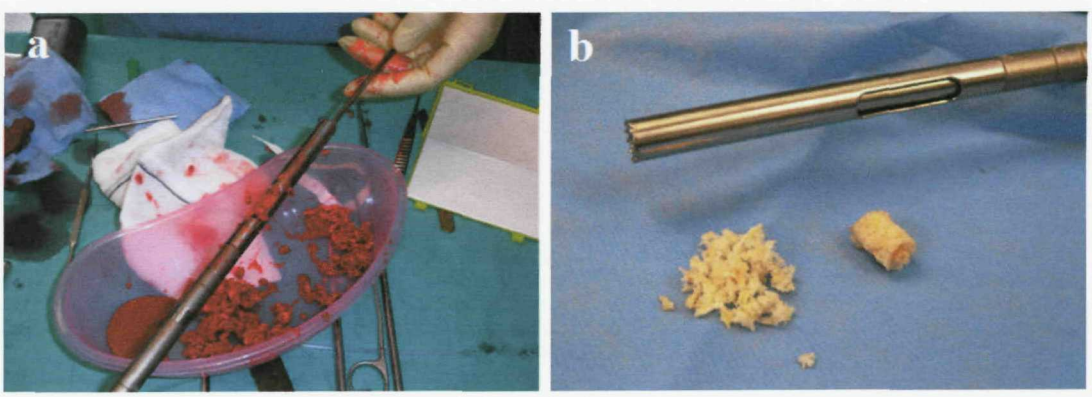


Figure 39. Seeded graft introduced into the corer and impacted ex vivo (a). In vitro laboratory simulation of graft impaction (graft pre- and post-impaction).

Representative samples including bone marrow aspirate, impacted allograft seeded with bone marrow stromal cells and allograft alone were taken for biochemical and histological analysis.

CASE 2

5.5 Introduction – Avascular necrosis of the femoral head

Avascular necrosis of the femoral head is a potentially devastating complication of intracapsular femoral neck fracture in the young patient. Surgical options for early disease (Ficat stage I and II) (Ficat, R. P. 1985) include core decompression and more recently implantation of autologous bone marrow cells (Gangji, V. et al. 2005a;Gangji, V. et al. 2005b;Gangji, V. et al. 2004;Hernigou, P. et al. 2002). Salvage procedures for advanced disease including the use of vascularised fibular grafts (Leung, P. C. et al. 1984) are technically demanding. Alternatives include the Bonfiglio strut graft (non vascularised), proximal femoral osteotomy to alter the pattern of stress transfer and vascularised pedicle flaps. Each procedure has substantial morbidity and to date there are no studies indicating the optimal procedure in the treatment of advanced disease and many patients will require early hip arthroplasty.

5.6 Case History

A thirty nine year old male presented with a six month history of pain and stiffness in his left groin and buttock. Eighteen months earlier he had fallen off his bicycle sustaining an intracapsular fracture of his left femoral neck. He was treated within twelve hours with a two hole Dynamic Hip Screw (DHS) and de-rotation screw and had made an uncomplicated post operative recovery remaining pain free for one year. The resultant pain was mechanical in nature and did not disturb him at night. On examination there was no obvious muscle wasting. Trendelenberg test was negative. There was no bony tenderness but internal rotation was reduced on the left.

Pelvis and left hip radiographs showed two defined areas of lucency in the left femoral head with apparent disruption of the articular surface and a degree of collapse of the femoral head (Figure 40 a and b).

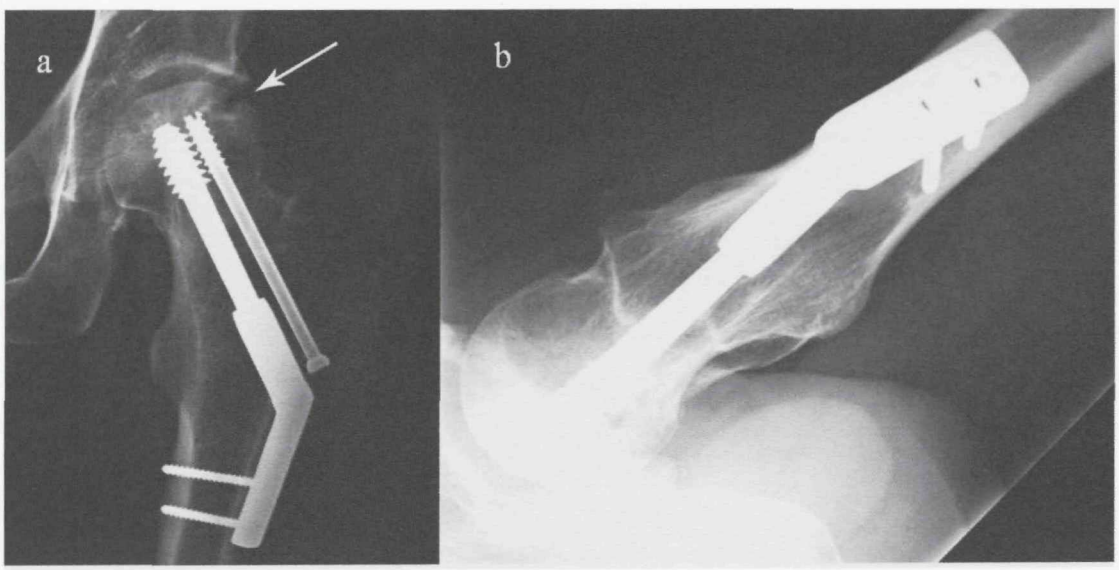


Figure 40. Anterior-Posterior (a) and Lateral (b) radiographs of Case 2 patient. Arrow denotes avascular destruction of the femoral head.

Due to the intrusive nature of the symptoms and the radiological evidence of avascular necrosis, it was decided to remove the metalwork and bone graft the femoral head and neck. Morsellised allograft, seeded with autologous marrow stromal cells was impacted into the deficient area. This study combined a combination biological and joint preserving approach utilising the positive attributes of decompression, osteoinduction/conduction and structural support.

5.7 Surgical Technique

A similar procedure to Patient 1 was performed. The patient was placed in the lateral position, prepared and draped. 50mls of bone marrow was aspirated from the posterior superior iliac spine and seeded onto prepared highly washed morsellised allograft obtained from 1 donated fresh frozen femoral head. The previous incision was used to expose and remove the dynamic hip screw and derotation screw. The screw tracks were curetted and under image intensification, the sclerotic bed was drilled. The seeded allograft was then impacted into the femoral neck using the 10mm cannulated femoral corer as before, without disruption to the articular cartilage. Samples were taken for biochemical and histological analysis.

5.8 Materials and Methods

Biochemical and histological techniques

Assay of Alkaline Phosphatase-Positive Colony-Forming Units. Bone marrow aspirate (2mls) taken intraoperatively was immediately plated, in theatre, into tissue culture flasks ($75cm^3$, n=3). 8mls of media (minimum essential medium – alpha modification,

10% foetal calf serum, 100uM ascorbate-2-phosphate, and 10nM dexamethasone) was added to each flask. The plates were incubated at 37°C in 5% CO_2. The media was changed including two washes with phosphate buffered solution (PBS) on the seventh day. On day 9 the samples were stained in situ for alkaline phosphatase activity. The number of alkaline phosphatase-positive colony forming units – fibroblastic (CFU-F) (colony is defined as containing more than 32 cells), were counted in each flask.

DNA and alkaline phosphatase-specific activity. Samples taken in theatre of impacted seeded allograft and were transferred to two, six well plates and media added. The plates were incubated at 37°C in 5% CO_2 and media changed every 4 days. Control samples of highly washed morsellised allograft were also cultured under identical conditions. At 1 week, constructs were washed with PBS, then incubated with Trypsin/EDTA (0·05%) at 37°C and 5% CO_2 interspersed twice with vigorous vortexing. Cells were collected by centrifugation (13,000rpm for 10 minutes at 4°C), and then resuspended in 1ml 0·05% Triton X-100. Lysis was achieved by freezing – thawing and samples were stored at -20°C until assayed. Lysate was measured for alkaline phosphatase activity using *p*-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1·5M, pH 10·3 at 25°C (Sigma, Poole, UK). DNA content was measured using PicoGreen according to manufacturer's instructions (Molecular Probes, Paisley, UK). Alkaline phosphatase-specific activity was expressed as nanomoles of *p*-nitrophenyl phosphate/hr/ng DNA.

Degree of graft compaction: A load cell of 5 kN capacity taken from an Instron 1173 materials testing machine (Instron Ltd., High Wycombe, Bucks) was used in the laboratory to measure the forces imparted to the morsellised graft during graft

compaction. The 10mm bone corer was loaded with representative amounts of wet washed allograft and the graft compacted by means of repeated blows to the plunger in the same fashion as in theatre. For each graft sample, three groups of ten blows were applied to the plunger and the peak and mean forces recorded. The mean and standard deviation of the force delivered per blow for three different surgeons were recorded.

5.9 Results

Clinical & Radiological

Both patients left hospital on day 1 post-operatively with simple analgesics and mobilised with protective weight bearing for 6 weeks, with two crutches whereupon both patients were reviewed and were both found to be asymptomatic. Radiographs at 12 weeks (Figure 41a-d), and computerised tomography slices at 6 months (Figure 41a and b inserts), showed satisfactory appearances of bone incorporation. In case 2, at 6 month follow up, there was no progression of AVN both clinically and radiologically.

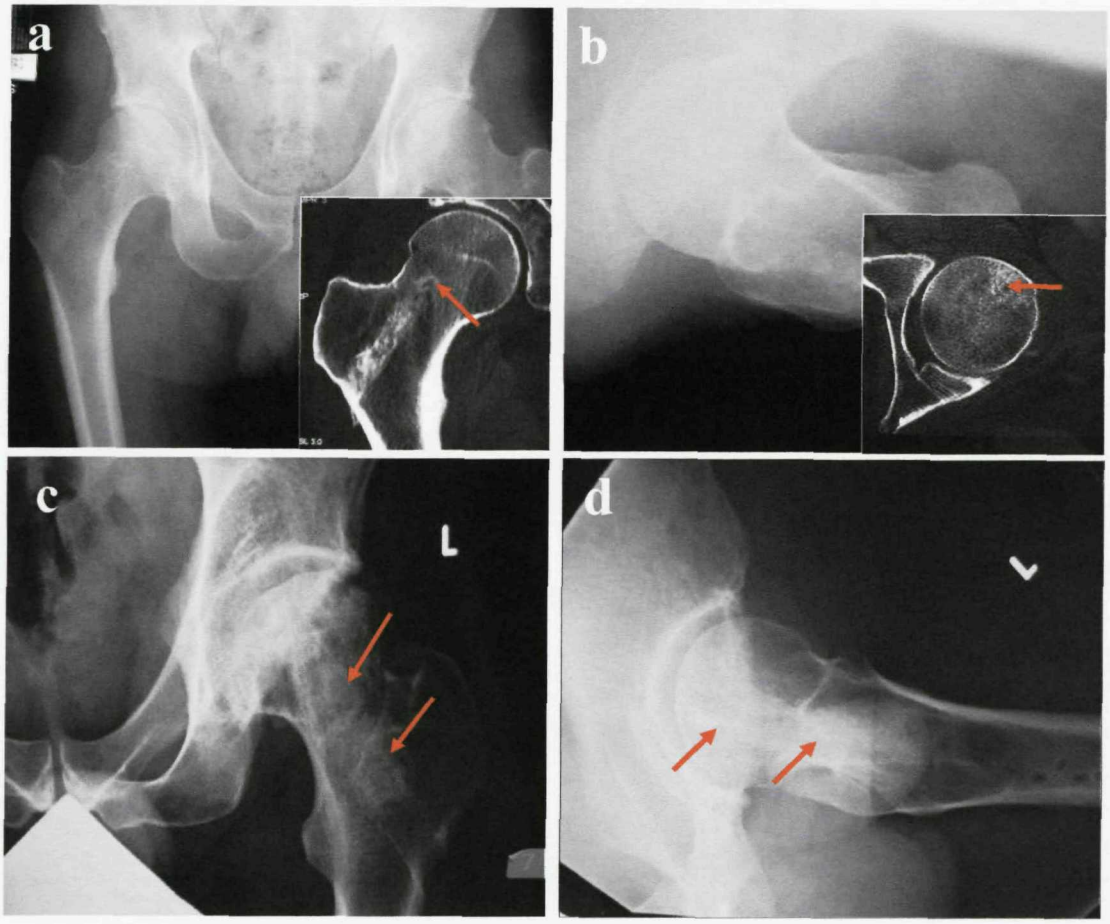


Figure 41. Postoperative Anterior-Posterior (AP) and Lateral radiographs of Case 1 (a and b). Images taken at 12 weeks are supplemented by Computer Tomography slices; Coronal (a, inset) and Axial (b, inset) at 6 months postoperatively. The lesion is seen to be replaced by bone at a higher density than the surrounding bone after impaction of the living composite. This feature is also seen in Case 2 (c and d) at 12 weeks.

Radiographs were again performed at the 18 month mark on both patients. Continued bony ingrowth and re-trabeculation was seen in both cases. This would suggest successful bony reconstitution in both the pathologically deficient areas and the drill tracks. In case 1 the superior cortex of the femoral neck would appear to have thickened and remodelled reducing the risk of fracture. In case 2 the subchondral bone

and articular surface defects in the femoral head have improved radiographically with smoothing of the articular surface compared to the pre-operative films.

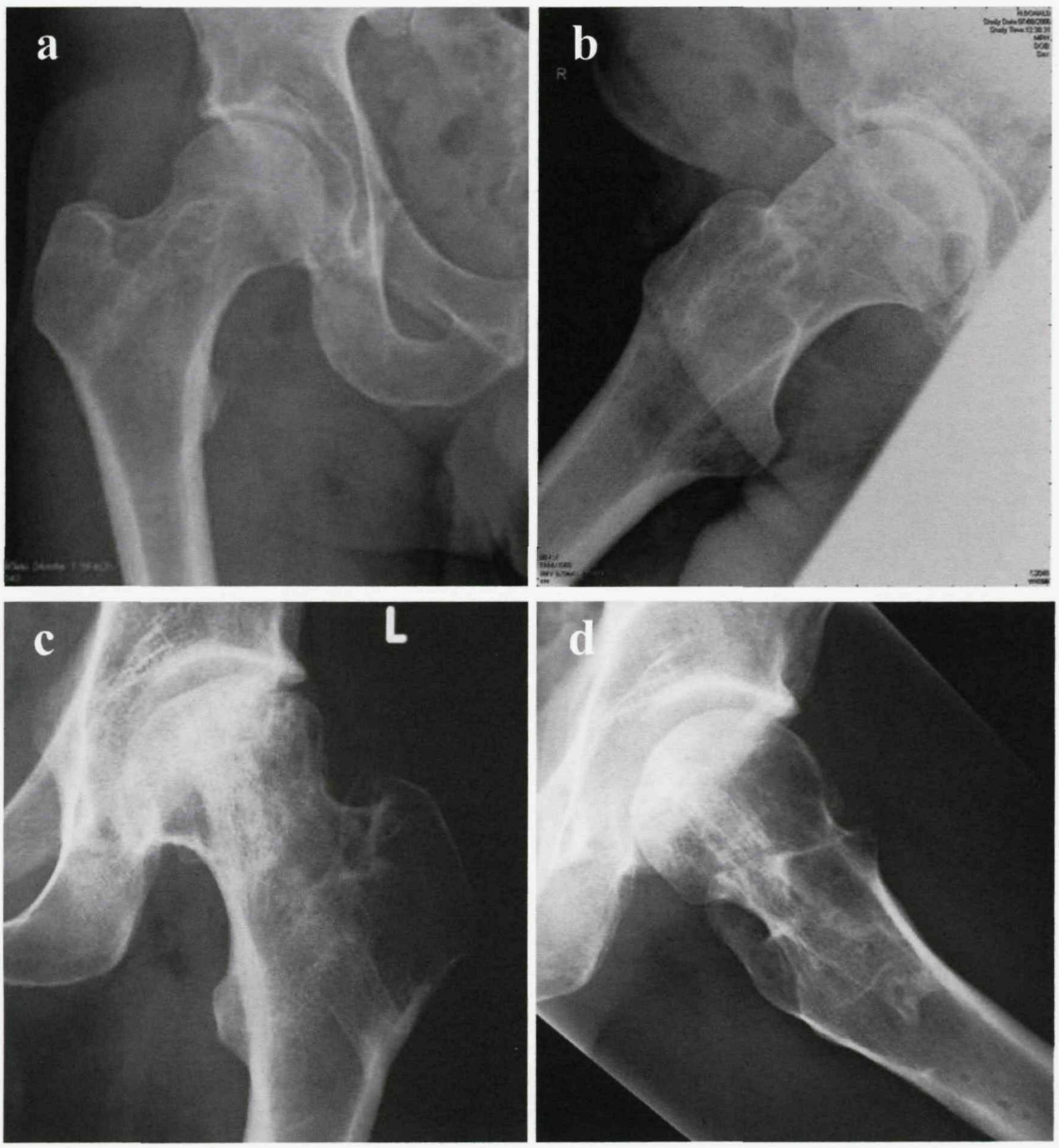


Figure 42. Radiographs at 18 months. Postoperative AP and Lateral radiographs of Case 1 (a and b). The grafted lesion and drill track has remodelled in certain areas and the superior cortex is not at risk of fracture. Case 2 (c and d) demonstrates similar features with smoothing of the articular surface.

Impaction Forces: The mean of the peak force delivered per strike to the graft was 0·7+/-0·13kN (SD) corresponding to average peak stresses within the graft of 8·3+/-1.5MPa (SD). There was no statistical difference between the three operators (Figure 43). This stress was greater in magnitude to our estimated peak stresses imparted in a standard femoral impaction bone grafting in revision hip surgery (0·8MPa). This was an expected finding as the pellet of allograft produced from the apple corer both in theatre and in the laboratory appeared more compact than impacted graft from a femoral impaction bone grafting.

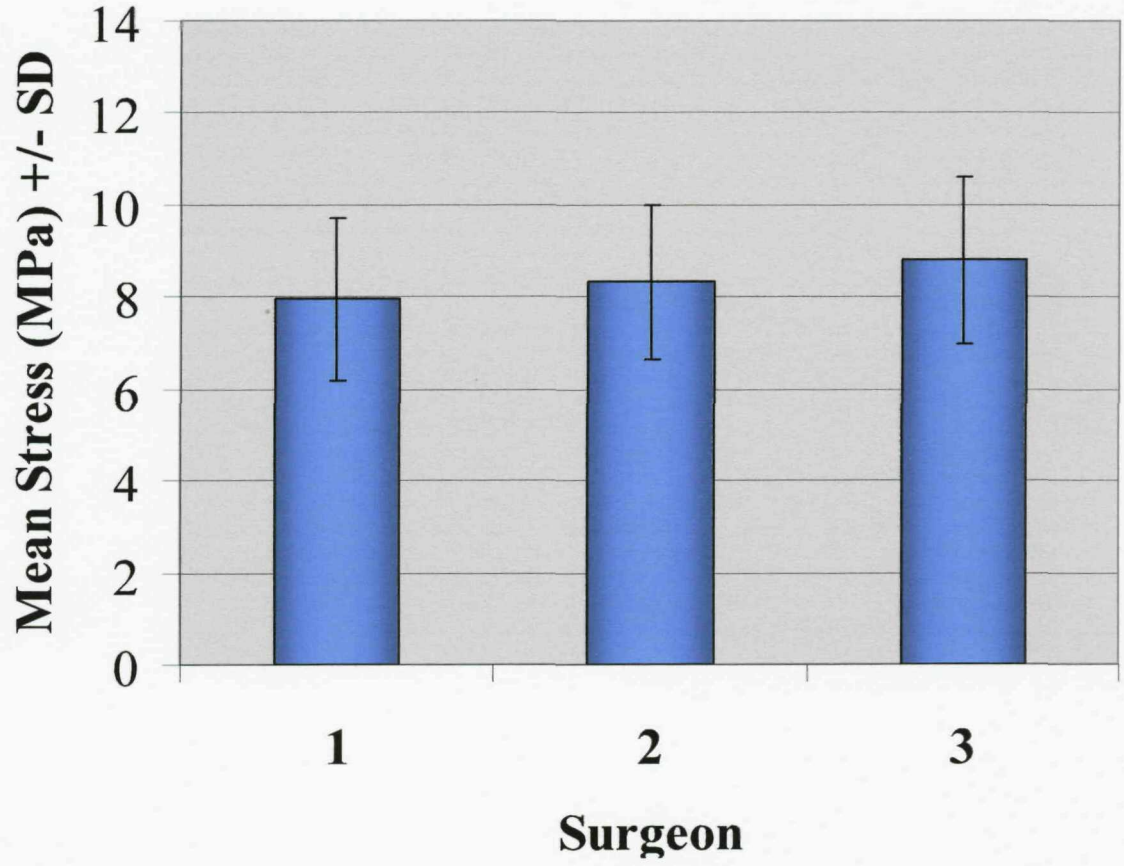


Figure 43. Mean peak stresses per strike imparted to the bone graft from 3 surgeons (n=3).

Alkaline Phosphatase-positive CFU-F and Enzyme activity: The bone marrow aspirate from case 1 yielded 4 x 10^5 nucleated cells/ml of aspirate and 2·75 x 10^6/ml from the patient in case 2. The average number of alkaline phosphatase-positive CFU-F formed was 2·18 (range 1·64–2·54) CFU-F/10^6 nucleated cells (fig 4a). The presence of large numbers of erythrocytes, in the absence of fractionation, in the theatre, resulted in a reduced surface area and suboptimal conditions for adherence and proliferation of the nucleated stromal cell population with a lower CFU-F yield. The exact mechanisms of reduced proliferation are not currently understood although expansion in the presence of high erythrocyte number appears a factor.

Biochemical: Samples from both cases of allograft seeded with bone marrow showed increased DNA content and specific alkaline phoshatase activity (Case 1, day 0: 0·05nM pNPP/hr/ngDNA Case 2, day 7: 23·7nM pNPP/hr/ngDNA) when compared to allograft alone (0nM pNPP/hr/ngDNA).

Histological: The histological analysis of the sample of tissue containing the cyst from theatre showed macro and microscopic appearances consistent with a non-ossifying fibroma. Immediate Ethidium Homodimer-1 / Cell Tracker Green live / dead staining confirmed cell viability post impaction (Figure 44b), and no cell activity from the allograft alone (Figure 44a). Repeat staining at 1 week demonstrated increased live cell numbers on the graft (Figure 44c).

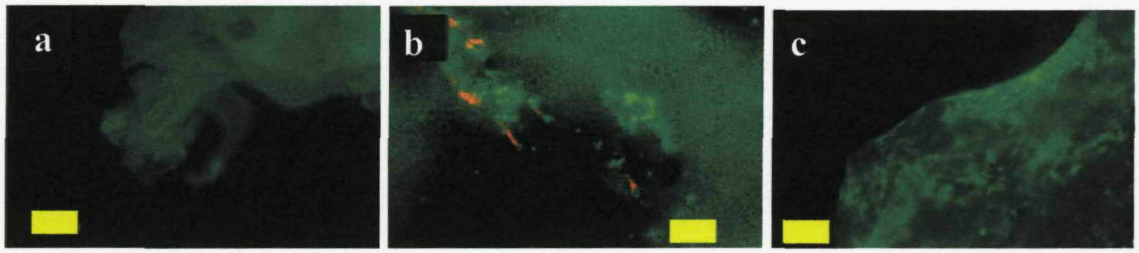


Figure 44. Ethidium Homodimer-1 / Cell Tracker Green staining of allograft alone (a). Immediate viability of seeded allograft (b). Viability of seeded allograft at 1 week. Magnification x5. Scale bar = 100 µm.

Staining with Haemotoxylin & Eosin showed dark staining marrow stromal cells adherent to the graft surface (Figure 45b). No cells were visible adherent to or surrounding the allograft alone (Figure 45a). Immunohistological staining was performed using Bone Sialoprotein (BSP). This is a major constituent of bone matrix, almost exclusively found in mineralized tissues and therefore considered a potential marker of bone metabolism (Stork, S. et al. 2000). Results of this staining demonstrated positive staining in a proportion of the marrow stromal cells at the allograft surface and in the surrounding haematoma. (Figure 45d).

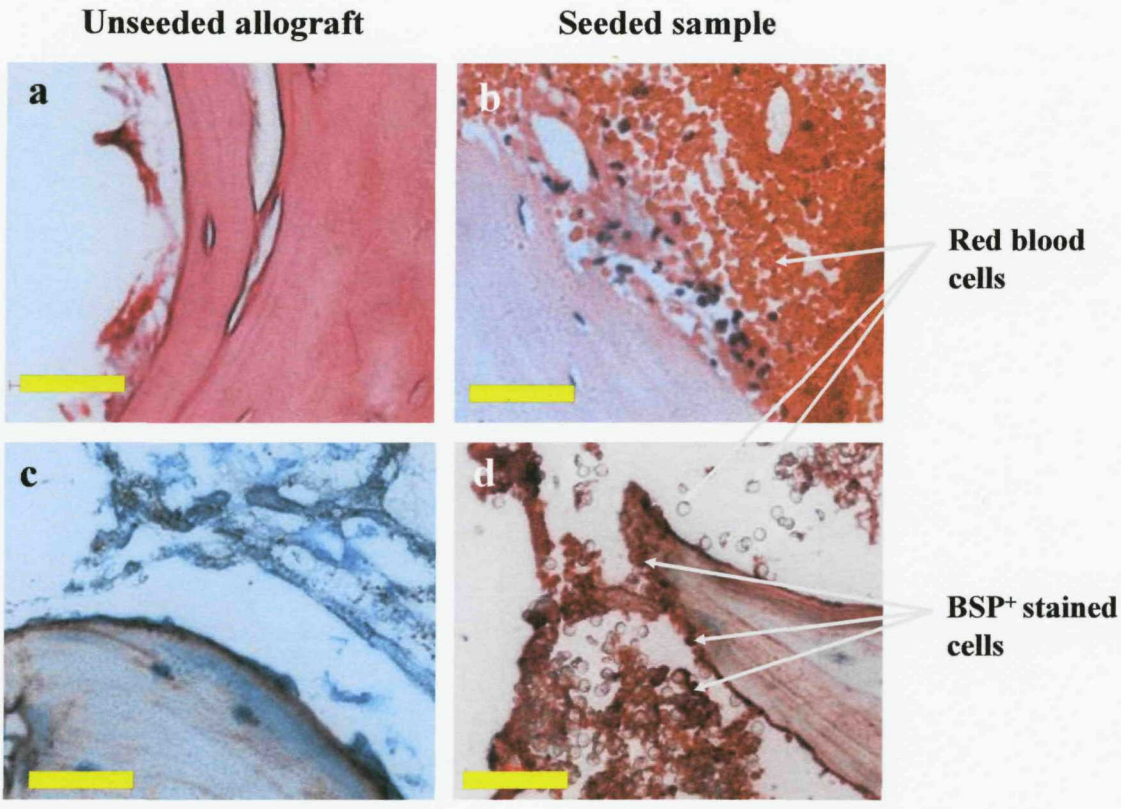


Figure 45. Haematoxylin and Eosin staining of Allograft alone (a) and Seeded Allograft (b). Bone Sialoprotein staining of Allograft alone (c) and Seeded Allograft (d). Original magnification x40. Scale bar = 50µm.

5.10 Discussion

This study demonstrates the potential to use autogenous human bone marrow cells for bone impaction and clinical potential therein. This study has demonstrated that autologous human bone marrow stromal cells can adhere to highly washed allograft, proliferate and express a bone phenotype after impaction into a contained defect in human subjects. In both cases bone marrow aspirate alone was used, in the absence of cell selection.

Central in this impaction procedure is the correct preparation of allograft to enable seeding of marrow stromal cells. In vitro data demonstrates that inadequate washing reduces adherence, survival and proliferative potential of seeded cells (Tilley, Bolland, Dunlop and Oreffo unpublished data). Sequential hydrogen peroxide washing followed by quenching in normal saline were used to remove the majority of lipid contaminants. Banked allograft remains a potential source of retained immunogenicity (and disease transmission). This can be attenuated by the process of freezing, however, the marrow elements within the cancellous bone, attributed to raising an immune response remain. In addition, removal of lipid and marrow fluid results in a stronger, compacted graft that displays greater resistance to shear (Dunlop, D. G. et al. 2003).

Histological analysis of washed allograft samples taken from theatre confirmed negligible or no bone marrow remnant within the graft infrastructure. Soil mechanical engineering principles have shown that a carefully graded and impacted aggregate will exhibit greater load carrying capacity than a poorly graded impacted aggregate (Lambe, T. W. et al. 1979;Smith GN 1990). Morsellised allograft has been combined with bone marrow to produce a living composite (Burwell, R. G. 1964a) but provides little structural support. However as seen in revision hip surgery, impacted bone graft can withstand significant loading.

In the current studies bone marrow stromal cells were observed not only to adhere to allograft but were able to withstand the forces of impaction and continue to proliferate offering significant potential in orthopaedic practice particularly in the field of revision hip surgery with impaction bone grafting. Improvements on bone marrow

fractionation to remove contaminating erythrocyte numbers will undoubtedly improve CFU-F yield, as observed in the laboratory.

Banked allograft is expensive and despite the robust septic screens and the aggressive intraoperative cleansing cycles there remains the potential for immune or septic reactions. However, the procedure, as advocated, carries minimal risk of donor site morbidity from the marrow aspiration. If multiple punctures of the cortex and small volume aspirations are performed there is also the risk of diluting the yield of osteoprogenitor cells (Muschler, G. F. et al. 1997).

This innovative impaction approach has a number of advantages:

1. The procedure fulfils the triad of osteogenesis, osteoinduction and osteoconduction with immediate effect.
2. It can be performed under a single anaesthetic without removal of tissue from the operating theatre thus avoiding issues of sterility associated with further expansion of marrow stromal cells.
3. The process provides a rapid, cost-effective, facile approach applicable to a number of clinical orthopaedic scenarios.

CHAPTER 6

6.1 General Conclusions

The results of three phases of this study have allowed us to reject the major null hypothesis which stated that "Bone marrow stromal fractions seeded onto washed morsellised allograft or synthetic β-TCP/PLA graft substitute will not survive and proliferate with a bony phenotype after impaction grafting." Survival and proliferation of marrow stromal cell fractions was demonstrated at several levels following a bone impaction procedure.

- The first study confirmed that HBMCs seeded on both washed allograft and PLA survived and proliferated with a bony phenotype following the physiological insult of a standard femoral impaction over a 17 day culture period. Confirmation of survival over a 24 hour period using a β-TCP scaffold allowed progression to Phase II.

- The second study demonstrated cellular viability and proliferation over a longer study period of 28 days both *in*-vitro and in vivo. Immunoselected bone marrow progenitor cells seeded onto synthetic β-TCP scaffolds and impacted would appear to produce a more successful living composite than unselected HBMCs on washed allograft.

- The third study involving human bone marrow aspiration, seeding of marrow mononuclear cells onto highly washed allograft and impaction of the living

composite were successfully adapted in a facile manner to the operating theatre to reconstitute areas of extensive bone loss.

These studies have demonstrated innovative techniques to aid the reconstitution of substantial bone loss when the volume of graft needed exceed that provided by autograft alone. HBMSC including those committed to the osteogenic lineage were seeded onto prepared allograft and synthetic scaffolds to create a living composite, as verified by laboratory analysis. Critically, the augmentation of graft with mesenchymal populations has been shown to survive the impaction process converting a loose aggregate into a viable live cell composite graft with comparable density to the cancellous bone that it has replaced without the need for additional metallic support and avoidance of infection.

This versatile technique can be tailored to a variety of orthopaedic situations and in particular offers an exciting potential role in the augmentation of allograft or synthetic graft, used in acetabular and femoral impaction bone grafting. Development of such incremental steps, utilising tissue engineering strategies is ongoing as a result of the initial success of the three studies discussed in this thesis. In particular biomechanical studies evaluating PLA behaviour are now at an advanced stage. It is hoped that further translation of such approaches will aid the unmet orthopaedic needs of many.

Chapter 7

7.1 Future Directions

Despite a historical paucity of clinical translation, tissue engineering has recently been exploring the use of scaffolds combined with HBMSCs in areas of the body that experience mechanical loading. Large bone defects up to 7 cm in length have been stabilised with HA seeded with HBMCs. The constructs demonstrated satisfactory biomaterial incorporation and bone formation (Quarto, R. et al. 2001). More recently, cell-based tissue engineering has been used to induce bone healing in a patient with longstanding refractory tibial non-union (Bajada, S. et al. 2007). This last study was of particular importance, using a synthetic calcium sulphate graft seeded with culture expanded HBMCs in a weight-bearing bone.

The combination of HBMCs seeded onto porous ceramics, especially those containing HA has often been suboptimal as a result of preferential bone ingrowth and calcification on the ceramic surface rather than true bone regeneration (Shin, M. et al. 2007). To this end further evaluation of non ceramic scaffolds such as PLA produced using $ScC0_2$ are particularly attractive. In addition to having a similar morphology and mechanical characteristics to cancellous bone, PLA can be manufactured to be resorbed over short time periods and to elute BMPs (Yang, X. B. et al. 2004;Schliephake, H. et al. 2007) and angiogenic growth factors (Kanczler, J. M. et al. 2007) at appropriate time points during hydrolysis thus aiding true bony regeneration. Potentially this could allow the surgeon to tailor the grafts resorption parameters to individual patients' needs. This could be hugely beneficial when planning surgery on patients at opposite ends of the age and reconstructive spectrum.

Formal mechanical testing of seeded impacted scaffolds will determine whether these constructs

Prior to clinical trials several factors need to be addressed, ideally with a large animal study. These include a loaded prosthetic bearing model to determine whether these constructs are stronger and support femoral prostheses at earlier time points. This would have obvious clinical implications with regard to early patient mobilisation and periprosthetic fracture risk. Additionally studies to confirm enhanced neovascularisation such as Positron Emission Tomography (PET scanning) and microangiography would be beneficial. The investigation of choice, both with large animal studies and subsequently in human trials would include a core biopsy to confirm whether enhanced remodelling and osseointegration occurred around the prosthesis when compared to those treated with unseeded graft alone. This latter issue will ultimately depend on the successful development of a local infiltrative vascular system. This form of retrieval study would need ethical approval as apart of a clinical trial in humans.

Large scale cellular expansion studies are needed to determine the time needed to generate therapeutic numbers of cells. Biosafety studies of the media and osteogenic additives in which the cells will be grown will also need evaluation. Work by Stute et al (Stute, N. et al. 2004) concluded that with regard to isolation and expansion, 10% autologous serum appeared at least as good as 10% FCS and avoids potential immunological, bacterial, prion and viral sequelae. It is feasible that following a simple bone marrow aspirate from the iliac crest and peripheral venous blood sample to provide the serum, MSCs could be expanded ex-vivo prior to seeding on an appropriate scaffold before impaction grafting surgery.

Current studies are centred on:

I) Improvements in the fractionation process to remove contaminating erythrocytes and increase CFU-F numbers.

II) Determination of the rate of bone incorporation.

III) In vitro culture of large scaffolds with osteogenic cells.

There has been recent interest with regard to seeding osteogenic cells on large or large volume three dimensional scaffolds. To this end, dedicated bioreactors have been developed and are showing encouraging results both with efficiency of adherence of cells to scaffold prior to implantation. The dynamic bioreactor systems described by (Olivier, V. et al. 2007) elegantly demonstrate how large volumes of graft can be successfully seeded with osteogenic cells and perfused ex vivo using a convergent flow technique prior to implantation. This method would be helpful to any upscaling of the techniques described in this thesis.

It would appear that the stage is now set for biological scaffolds in combination with culture expanded autologous cells as an attractive surgical proposition in bone impaction grafting. Totally synthetic scaffolds with similar or improved mechanical properties to impacted allograft, seeded with progenitor cells without the potential for disease transmission or immunological complication may provide a facile tissue engineering scaffold. This would appear to be an important advance in clinical tissue engineering for bone loss.

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APPENDICES

Appendix 1 – Laboratory techniques

STRO – 1 fraction isolation

STRO-1+ marrow stromal cells from human bone marrow were immunoselected with the antibody STRO-1, utilising a magnetically activated cell separation (MACS) system as described previously (Howard, D. et al. 2002). Following the same technique for preparation and centrifugation for marrow cells as described above, red blood cells were then removed via centrifugation with Lymphoprep solution. Nucleated cells were resuspended at 1x10^8 cells per 10ml of blocking solution (HBSS with 5% FCS, 5% human normal AB serum and 1% BSA fraction v) then incubated with the STRO-1 antibody hybridoma for 1 hr. After washes with MACS buffer (HBSS containing 1% BSA fraction V) cells were incubated with MACS anti-IgM beads for 45 mins, before passing the cells suspension through a MACS column in the presence of a magnet to give the negative fraction. The column was then washed and in the absence of the magnet 1ml MACS buffer was passed through the column to yield the STRO-1+ fraction. The culture was maintained in osteogenic medium until confluent, then passaged to well plates for expansion.

Live / Dead staining

To demonstrate cell viability, cells were labelled with 10μg per ml Cell Tracker Green™ CMFDA and 5μg/ml Ethidium Homodimer-1 (CTG/EH-1) to label viable and necrotic cells respectively. Prior to fixation, cells were bathed in α-MEM

containing CTG/EH-1 at 37°C for 1 hr. Samples were washed twice with α-MEM, then bathed for a further 60 mins in α-MEM only. Samples were rinsed in PBS before fixing in 70% ethanol for 15 mins. Samples were immersed in PBS and visualised with appropriate fluorescent filters.

Fixation and paraffin embedding

Samples for alkaline phosphatase staining were fixed in 95% ethanol and CTG/EH-1 labelling at 70% ethanol, all remaining samples were fixed with 4% paraformaldehyde. Samples embedded in paraffin wax were first dehydrated in 1 hr graded ethanol stages of 50%, 90% and 100% (absolute) ethanol. Following a 15 min soak in 1:1 ethanol/chloroform solution, samples were placed in chloroform twice for 1 hr then soaked in paraffin wax at 60°C twice again for 1 hr. Samples were embedded in hot wax and placed on a cooling surface at 2–4°C to solidify. For embedding of biodegradable polymer scaffolds (e.g. poly-lactic acid), histoclear was substituted for the chloroform steps as chloroform was found to dissolve the polymer scaffolds. Paraffin wax embedded blocks were stored at 4°C prior to sectioning.

Methylmethacrylate embedding

All steps of fixation, dehydration, and infiltration were carried out at 4C on a magnetic stirrer. The tissue samples were fixed in 40% ethanol for 48 hrs. Samples were dehydrated according to the following schedule: 70% ethanol (2 days), 95% ethanol (2 days), 100% 2-propanol (twice for 1 day), and xylene (twice for 2 days). After dehydration, the samples were infiltrated with the plastic embedding mixture

using a three-step protocol. In each MMA solution, the samples were infiltrated for 3 days. MMA Solution I consisted of 60 ml MMA (Merck; containing 100 ppm hydrochinon) + 35 ml butylmethacrylate (Sigma; containing 10 ppm hydrochinon) + 5 ml methylbenzoate + 1.2 ml polyethylene glycol 400. MMA Solution II was a mixture of 100 ml MMA I with 0.4 g dry benzoyl peroxide, and MMA Solution III consisted of 100 ml MMA I + 0.8 g dry benzoyl peroxide. All MMA solutions were stirred for at least 1 hr before use. The infiltrated tissue was placed on a polymerized layer of plastic in the bottom of the moulds. To prepare the plastic bases, 600 µl of *N,N*-dimethyl-*p*-toluidine was added to 100 ml of cold (4C) MMA III and stirred for a few minutes. Immediately thereafter, 5 ml of the polymerization mixture was poured into each mould. To prepare the polymerization mixture, 400 µl of *N,N*-dimethyl-*p*-toluidine was added to 100 ml of cold (4C) MMA III, and stirred for a few minutes. After addition of the accelerator, care was taken that the polymerization mixture was kept cold at all times. After the infiltrated tissue was placed on the plastic layer in the vials, the vials were completely filled with polymerization mixture (about 20 ml) to exclude air, capped, and transferred to a deep-freezer. Polymerization was carried out at -18 to -20C and was complete within 3 days. Polymerized blocks were stored at -20C.

After trimming of the plastic blocks, 3-5-µm thick sections were prepared at room temperature with a microtome equipped with a tungsten carbide cutting edge. During sectioning, the knife and the blocks were kept moist with 30-40% ethanol. The sections were transferred onto chromalum-gelatin-coated slides and carefully stretched using 70% ethanol. Thereafter, the sections were covered, pressed with a slide press, and dried for 2 days.

For deplasticization, the sections were placed in three changes of 2-methoxyethylacetate for 20 min each, two changes of acetone for 5 min each, and two changes of deionized water for 5 min each. Sections were routinely stained with toluidine blue at acid pH.

Slide preparation

Paraffin wax embedded samples were removed from cold room storage and trimmed of excess wax. Using a Microm 330 microtome (Microm International GmbH, Germany), sections were cut at 6μm and placed on a water bath at 37°C and allowed to spread. Sections were transferred to pre-heated glass slides on a 37°C hotplate and left for 30 mins. Slides were moved to a drying oven at 37°C for 2–3 hrs then stored at 4°C. Glass slides were warmed to room temperature prior to staining.

Alcian blue and Sirius red staining

Excess wax covering the sections was removed via washes in histoclear then samples were rehydrated through graded methanol stages of 100%, 90% and 50%. Sections were soaked in a water bath then followed by nuclear counter-staining with Weigert's haematoxylin for 10 mins. Slides were rinsed in water, followed by 0.5% Alcian blue 8GX staining for 10 mins to demonstrate proteoglycan-rich cartilage matrix. After 20 minutes pre-treatment with 1% molybdophosphoric acid, sections were stained with 0.1% Sirius red F3B for 1 hr to demonstrate collagen, then dehydrated through reverse graded methanol (50%, 90%, 100%) and histoclear steps. Sections were

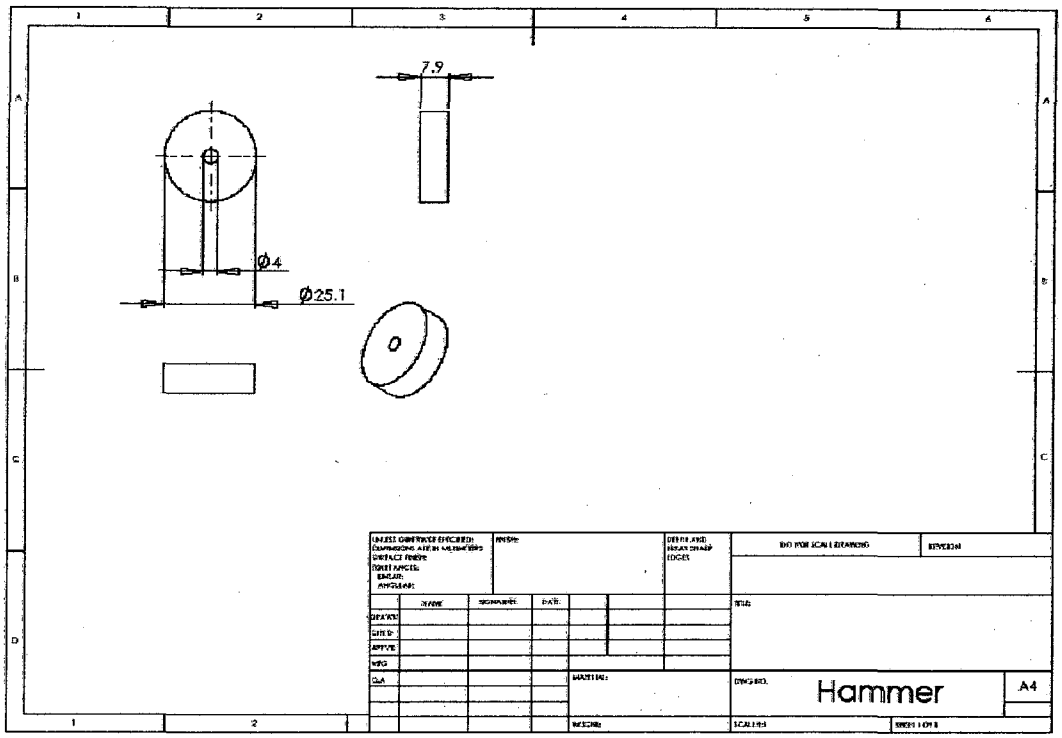
mounted with dibutyl phthalate xylene (DPX) underneath glass coverslips.

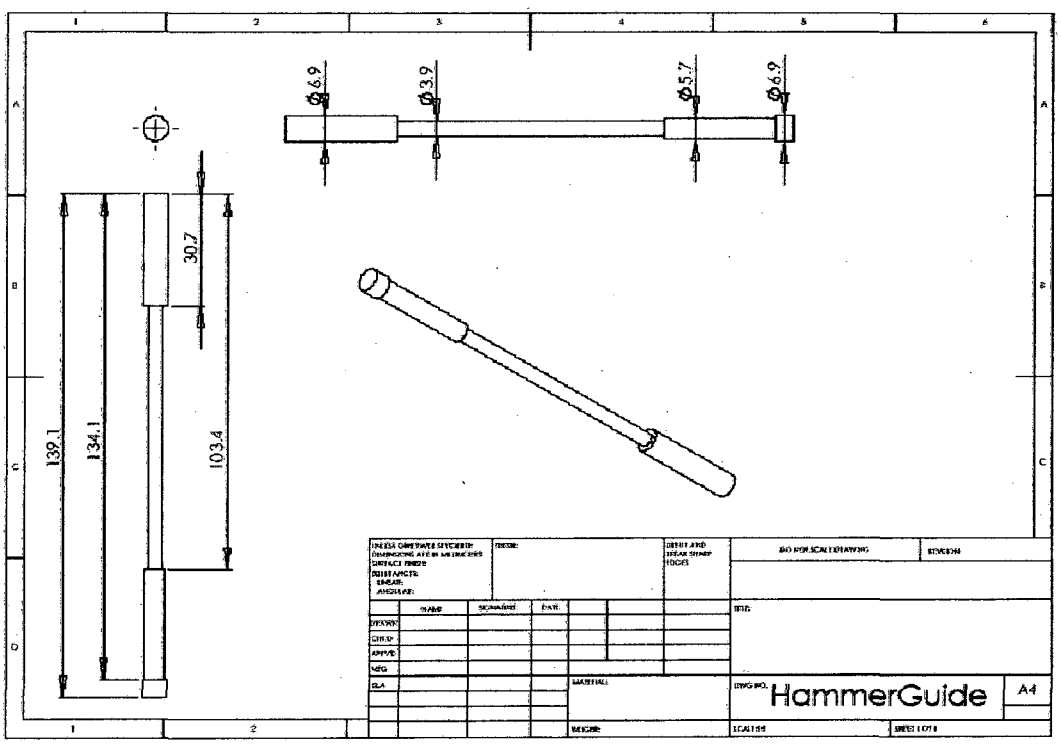
Immunocytochemistry

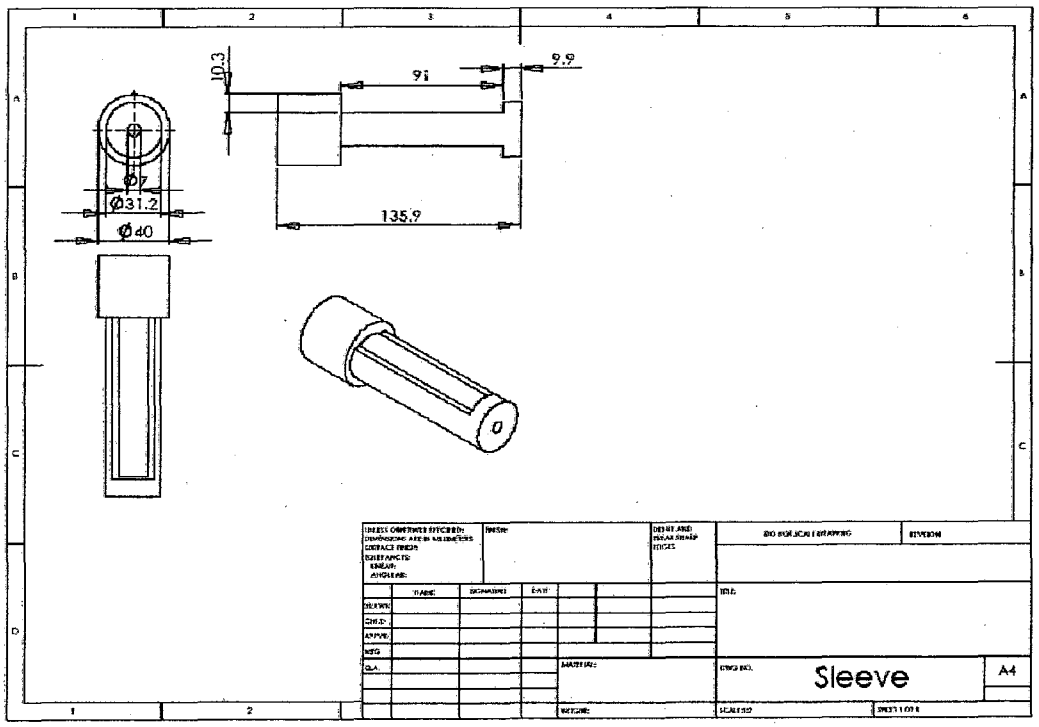
Excess paraffin wax was removed as described above and slides were rinsed in the water bath. After quenching endogenous peroxidase activity with 3% H2O2 for 5 mins, sections were rinsed with water and blocked with 1% BSA in PBS for a further 5 mins. Slides were drained and sections were incubated overnight at 4°C with the primary antibody. Sections were rinsed with water and taken through wash buffers of high salt (1M NaCl, 50mM tris, 0.05% tween), low salt (0.5M NaCl, 50mM tris, 0.05% tween) and tris (0.1M tris, 0.1% tween) for 5 mins each to remove residual antibodies. The appropriate biotin-conjugated secondary antibody was applied for 1 hr followed by further rinses in wash buffers. Streptavidin peroxidase was linked to the secondary antibody complex following 30 min incubation then rinsed again in wash buffers. Samples were developed using 3-amino 9 ethyl carbazole (AEC) in acetate buffer containing H2O2, to yield a reddish-brown reaction product, and then mounted with glycerol jelly. Negative controls either lacked the respective primary antibodies or were incubated with appropriate isotype controls, and no staining was observed in negative controls.

Blocking and primary antibody incubation periods were as described above for LF67 type I collagen antibody (1:300) with anti-rabbit IgG biotin-conjugated secondary antibody (DAKO; 1:200 with 1% BSA in PBS). Type I collagen polyclonal antibody was a gift from Dr Larry Fisher, NIH, Bethesda, USA.

Appendix 2 - Technical drawings of graft impactor



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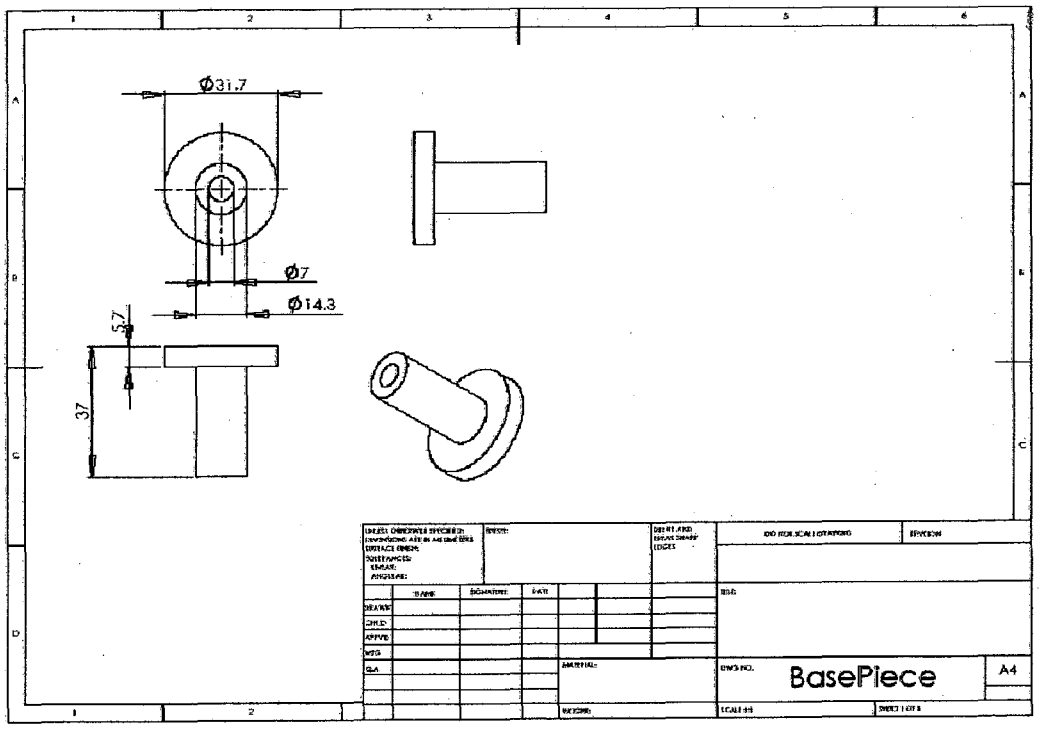
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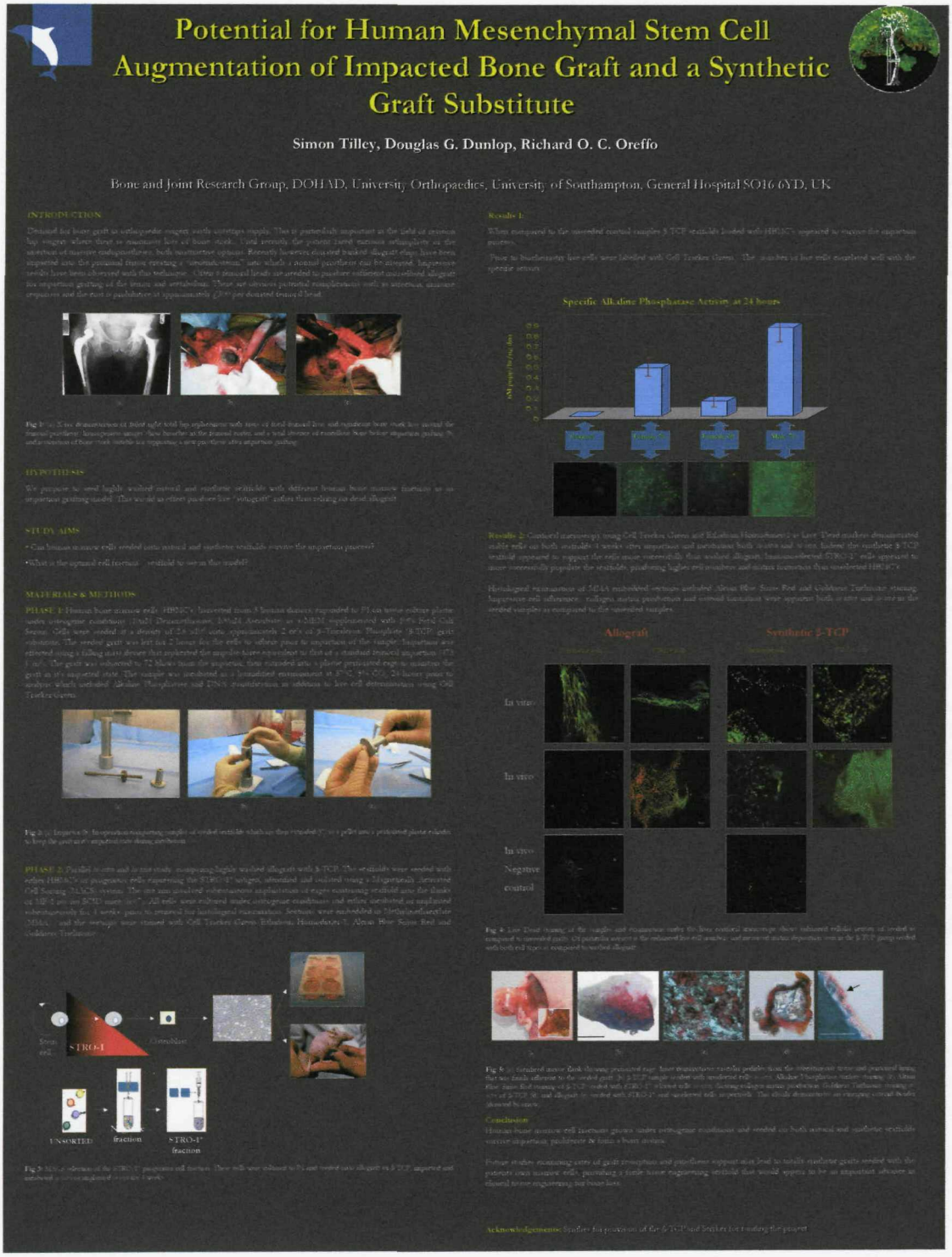
Appendix 3 – Clinical Science poster prize, Bone and Tooth Society (BATS) Annual Meeting 2005



Appendix 4 – Large animal study proposal

Null Hypothesis: "At 12 weeks after implantation, there is no difference in radiographic migration, mechanical competence or biological integration of a femoral prosthesis when implanted into an ovine model as follows:

(1) Allograft with culture expanded stromal cells.

(2) 50:50 Allograft / PLA with culture expanded stromal cells.

(3) 50:50 Allograft / PLA,

compared to sheep allograft alone.

Calculation of sample size

The sample size required to test the hypothesis is based on an estimate of expected subsidence within the first 12 weeks of implantation. There is large variance of quoted subsidence for human impacted grafted total hip replacements with a range of average subsidence from 1 – 9 mm within the first year, with individual subsidence ranges up to 37 mm. The sheep model is believed to equate to experiences in hmans. Subsidence of the control group (allograft) is anticipated at 1 mm +/- 1 mm standard deviation. A clinically significant difference is estimated at 2.5 mm.

The Type I and Type II errors were assigned as $\alpha = 0.05$ and $\beta = 0.2$ (2 tailed, 95% confidence with 80% power). The sample size can be calculated using the above figures with the NHMRC (National Health & Medical Research Council) sample size calculation software (SAM). The number of animals determined for each group was 8. To be specific, with 2 groups of 8 sheep there is 80% power to detect a difference

between the means of 1.51 standard deviations at the 5% significance level, and 90% power to detect a difference of 1.75 SD's.

Review of the relevant literature of sheep hip arthroplasty suggests a 10% complication rate might be expected. Data from Dunlop et al indicates a complication rate of 20% for the total hip replacement model and 10% for the hemiarthroplasty model. The revised sample (N') to account for dropouts was obtained by the following formula:

$N' = N / (1 - R)^2$

N' = Revised sample rate; N = first estimate sample size; R = drop - out rate.

$N' = 8 / (1 - 0.1)^2 = 9.87$

Therefore 10 animals are proposed for each group.

Inclusion, Exclusion Criteria, Stratification and Randomisation

Animals will be assessed for physical fitness prior to inclusion in the study by an independent veterinary surgeon.

Randomisation will be performed using blank sealed envelopes containing an enclosure with the proposed treatment option written on it. Ten of each enclosure will be made, sealed and shuffled independently. Each animal will be assigned a study number and an individual identification number.

Appendix 5 – Publications

Adult mesenchymal stem cells and impaction grafting: a new clinical paradigm shift. (Review article)

Bolland B J, **Tilley S**, New A M, Dunlop D G, Oreffo R O C

Expert Rev Med Devices, 2007 May; 4(3):393-404.

Taking tissue-engineering principles into theatre: augmentation of impacted allograft with human bone marrow stromal cells.

Tilley S, Bolland BJ, Partridge K, New AM, Latham JM, Dunlop DG, Oreffo RO

Regen Med 2006 Sep; 1(5): 685-692.

Adult mesenchymal stem cells and impaction grafting: a new clinical paradigm shift

Benjamin JRF Bolland, Simon Tilley, Andrew MR New, Douglas G Dunlop and Richard OC Oreffo[†]

The demographic challenges of an increasingly aging population emphasize the need for innovative approaches to skeletal reconstruction to augment and repair skeletal tissue lost as a consequence of implant loosening, trauma, degeneration or in situations involving revision surgery requiring bone stock. These clinical imperatives to augment skeletal tissue loss have brought mesenchymal stem cells to the fore in combination with the emerging discipline of tissue engineering. To date, impaction bone grafting for revision hip surgery is a recognized technique to reconstitute bone utilizing morselized allograft to provide a good mechanical scaffold, although with little osteoinductive biological potential. This review details laboratory and clinical examples of a paradigm shift in the application of mesenchymal stem cells with allograft to produce a living composite using the principles of tissue engineering. This step change creates a composite that offers a biological and mechanical advantage over the current gold standard of allograft alone. This translation of tissue engineering concepts into clinical practice offers enormous input into the field of bone regeneration and has implications for translation and future change in skeletal orthopedic practice in an increasingly aging population.

Expert Rev. Med. Devices 4(3), 393–404 (2007)

CONTENTS

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KEYWORDS:
allograft, impaction bone grafting, mesenchymal stem cells, translational research

Burden of the problem

Total hip replacement (THR) remains one of the most successful procedures performed by any surgeon, as measured by quality adjusted life years with more than 90% clinical success rate at 10 years [1]. Each year in the UK there are over 50,000 THRs (1 million worldwide) performed at a cost of approximately £250 million per annum. With the demographics of an aging population, this figure is set to rise by as much as 50% by 2026 [2]. At the opposite end of the age spectrum, the combination of younger and more active patients with higher expectations and surgeons prepared to perform surgery on this cohort has further exacerbated the growing demand for THR surgery [3]. This cohort will place higher demands on the prosthesis for a longer period of time, further increasing the likelihood of revision surgery. In consequence, revision hip surgery has increased by 100% due to implant failure since 1991 [3] and figures from the American Academy of Orthopaedic Surgeons estimate that this rise will continue at a rate of 20–30% over the next 30 years.

There are a number of issues surrounding revision hip arthroplasty, namely [4,5]:

- Revision operations are expensive
- Have a higher complication rate
- Longer operative time
- Such procedures typically have significant blood loss in comparison with primary procedures
- Outcomes are often inferior to the primary operation

The cause of many of these difficulties is a result of the extensive bone loss associated with loose implants requiring revision surgery. When an implant fails, bone is lost through a combination of stress shielding, osteolysis, instability and/or infection. The surgical

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treatment of extensive bone loss and voids fall into the categories of replacement, with cement or custom-made prostheses, or reconstruction using the technique of impaction bone grafting (IBG). With primary THRs and subsequent revision THRs being performed on younger patients, restoring rather than replacing bone stock will be the chosen option.

Impaction bone grafting: origins to the present day

The technique of IBG in the acetabulum was first introduced by Slooff and colleagues in Nijmegen, The Netherlands [6] in the late 1970s, and later in the UK (in the femur) by Ling, Gie and colleagues in Exeter in 1987 [7]. The procedure involves the progressive compaction of morselized allograft into contained femoral and/or acetabular cavities, into which the prosthesis is cemented. This generates a four-layer composite of host bone, allograft bone, cement and prosthesis.

The objective of IBG is to initially provide bone support and stable fixation for the implant – essential for successful clinical outcome – as well as subsequent bone ingrowth and remodeling of the impacted allograft with subsequent restoration of living bone stock. Thus, the success of IBG is dependent on both biological and mechanical factors.

Although results from the center of origin have shown excellent mid-term outcome, with 99% survival at average 10 year follow-up (in 226 patients with a femoral reoperation due to symptomatic aseptic loosening as the end point) [8], this has not been the experience of other centers. In Bristol, in a group of 79 hip replacements followed up for just over 1 year, in whom impaction grafting of the femur had been performed, nine (11%) demonstrated evidence of significant subsidence [9]. This was defined as subsidence of over 10 mm and in all cases occurred in the first 3 months postoperatively. Six hips required subsequent re-revision. A series in Australia found similar subsidence values of 9 mm (range 2–37 mm) compared with cemented revisions at 24 months [10]. Potential factors responsible for the variation include etiology of failure of the primary THR, implant choice, surgical technique and bone graft.

In terms of graft material of choice, morselized allograft remains the current gold standard in IBG. Although allograft acts as an adequate mechanical scaffold, it is primarily a nonviable necrotic tissue with limited osteoinductive potential. Other concerns surrounding the use of allograft include the potential for immunogeneic response or disease transmission. Since the evolution of allograft for IBG, many studies have investigated techniques to improve the mechanical and, to a lesser extent, biological properties of allograft. These are described below.

Preservation of the graft

Bone graft is normally obtained from femoral heads from live donors at THR surgery. The femoral heads are either fresh frozen at -80°C or freeze dried and stored at room temperature. Freeze-dried bone reduces the immunogeneic load and risk, although it has been shown to have inferior mechanical properties in comparison with fresh-frozen bone [11,12]. However, *in vitro* impaction studies comparing freeze-dried and fresh-frozen morselized allograft have shown that both reach similar maximum stiffness levels (55 MPa); however, the freeze-dried graft required fewer impactions [13]. To date, the evidence favoring one form of preservation over the other remains unclear, however, the majority of surgeons, particularly at the centers where the technique originated, continue to use fresh-frozen allograft.

Graft preparation (grading, washing)

The techniques employed to prepare bone graft have been shown to affect both the biological and mechanical properties of the graft and play a key role in successful outcome. Studies have utilized soil mechanics theory of aggregates [14,15] to measure the shear strength of morselized allograft. In IBG, allograft will fail in shear and therefore the measurement of shear strength and its individual components have been recognized as important properties of the graft. Briefly, the shear strength (τ) of a granular aggregate, such as bone graft, depends on the internal friction (Φ), expressed as the angle at which the aggregate will slide and interlocking of the particles (c), expressed as a stress. The frictional resistance varies in proportion with the normal (compressive) stress (σ) produced by the load supported by the aggregate. The relationship between the parameters can be expressed by the Mohr Coulomb failure law:

$$\tau = c + \sigma \tan \Phi$$

which allows us to calculate the shear strength of an aggregate. Studies by Dunlop and colleagues measured these parameters in bone graft to determine the effect that particle size and washing had on the shear strength of the graft [16]. They concluded that a more compacted graft can be produced if the graft is made up of a broad range of particle sizes rather than a narrow range. They also showed that washing improved the shear strength of the graft. By removing the fat and marrow, with washing, there is also a theoretical additional benefit of reducing the immunogeneic load to the patient. On the contrary, washing will also remove key osteoinductive agents and therefore could be seen as detrimental.

Bone-graft extenders

Bone graft alone, either morselized or whole, has had some success in replacing lost bone stock [6,17]; however, with demand outweighing limited supply and the increasing concerns regarding transmission of pathogens, interest in synthetic materials has increased significantly.

In IBG, synthetic grafts, such as allograft, have a structural, as well as an osteoconductive, role. The synthetic materials or bone-graft extenders, thus, must initially provide sufficient mechanical support for the prosthesis. Thereafter, the material is required to provide a framework onto which the host bone and vascular network can regenerate and heal. In addition, the graft should also interact with the host tissue, recruit and, ideally, promote differentiation of osteogenic stem cells, rather than just behaving as a passive scaffold framework. These synthetic grafts are resorbed or degraded at various rates altering

the mechanical properties of these materials with time (particularly integral to IBG where the graft plays such a major mechanical role). The mechanical properties are also influenced by synthetic graft morphology. A macroscopic open structure that interdigitates within the composite, analogous to reinforced concrete, crossing multiple potential shear planes would provide further resistance to shear and composite failure. Two groups of synthetic material identified as potential bone-graft extenders in IBG include ceramic and polymeric scaffolds.

Ceramic calcium phosphate-based materials include β-tricalcium phosphate (β-TCP), hydroxyapatite (HA) and biphasic mixtures of the two components, all of which are considered to be usable as bone-graft substitutes. TCP is an excellent osteoconductive material as it initiates bone formation. However, TCP is readily resorbed by osteoclasts and, therefore, is typically used in non-load-bearing situations. By contrast, HA displays a slow rate of resorption but is brittle and mechanically weaker as a bone host until invaded by host tissue. To date, the slow resorption is considered to be a disadvantage in a number of clinical settings and, as a result, several modifications have been made to increase the rate of resorption, including the generation of biphasic composites of HA and β-TCP.

In vitro and *in vivo* studies using TCP/HA extenders in an IBG model have, interestingly, shown enhanced mechanical stability over allograft alone [19,20]. This may be explained by soil mechanics theory, whereby the smaller particles fill the gaps left between the allograft particles increasing interparticulate cohesion and shear strength (see Mohr Coulomb failure law). Graft incorporation of TCP/HA particles has been observed in a goat model of contained acetabular deficiencies, reconstructed with a 50:50 mix of graft and extender [21].

Polymeric scaffolds, including synthetic materials, such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and polyglycolic acid, have attracted significant interest in the tissue engineering community as a consequence of their biocompatibility, the ease of processing into 3D structures, their established safety as suture materials and the versatility that they offer for producing chemically defined substrates for bone-graft matrices. These degradable materials alone display little osteoconductive potential, however, these synthetic materials can be prepared under conditions that do not denature peptide growth factors, allowing these factors to be integrated within and delivered by the polymer in, potentially, an appropriately orchestrated temporal fashion via hydrolysis [22]. The time-release delivery of inductive agents could accelerate bone regeneration augmenting both the biological and mechanical integrity of PLA. Initial *in vitro* and *in vivo* studies exploring the biocompatibility and mechanical properties of PLA and, therefore, its potential role as a bone-graft extender in IBG, are described later in this review.

Bone-graft additives

Although impaction is mechanically beneficial it has been shown to be detrimental to bone ingrowth and remodeling [23]. This has led to widespread interest in graft additivies and, in particular, the application of bone morphogenetic proteins (BMP), such as BMP-7 or osteogenic protein (OP)-1. In a bone chamber animal model, Tagil and colleagues demonstrated improved initial graft resorption and hastened graft incorporation and remodeling after the addition of OP-1 [24]. However, OP-1 not only stimulates new bone ingrowth but also simultaneously activates osteoclastic activity. McGee and colleagues reported increased resorption in allograft containing OP-1 compared with allograft alone at 6 weeks in a sheep femoral impaction model associated with stem subsidence [25]. This is probably attributable to stimulation of osteoclasts by OP-1. The temporal relationship between bone resorption and regeneration is clearly critical in the clinical scenario of IBG when the graft is to be loaded from the outset. Gaining control of this fine balance has led to studies utilizing the role of bisphosphonates in inhibiting bone resorption. Aspenberg and Astrand demonstrated that the addition of a bisphosphonate improved bone-graft density but also reduced the bony ingrowth when compared with OP-1 alone [26].

It is clear that despite all of these studies little progress has been made on the biological augmentation of allograft. Critically, the allograft used in IBG is nonvascularized and it is therefore unclear how successful incorporation is achieved. Histology from biopsy samples has shown viable tissue ingrowth into the graft [27], but confirmation of new bone formation within the defects remains limited and, at best, inconsistent [28–30].

The expanding emerging discipline of tissue engineering has opened an exciting avenue to improve both the biological and mechanical aspects of allograft in IBG for revision hip and knee surgery.

Tissue engineering & impaction bone grafting

The general principle of tissue engineering involves the combination of living cells within a natural or synthetic scaffold to produce a 3D living tissue construct that is functionally, structurally and mechanically equal to, if not superior to, that which it has been designed to replace [31].

There are a number of different sources of cells that can be used for tissue repair and regeneration. These include mature cells from the patient, 'adult' stem cells, such as human bone marrow stromal cells (HBMSCs) or mesenchymal stem cells (MSCs), as well as fetal and embryonic stem cells. This review will concentrate on the role of adult stem cells in IBG.

The regenerative capacity of bone has long been recognized, although the existence of a multipotent MSC has proved elusive owing to the low incidence, indeterminate morphology and undefined biochemical phenotype of these cells. Evidence for a population of cells with multilineage mesodermal differentiation capacity was first demonstrated by Friedenstein and colleagues in studies that showed the capacity of clonogenic fibroblastic precursor cells (CFU-F) to generate cartilage, bone, myelosupportive stroma, adipocytes and fibrous connective tissue (FIGURE 1) [32–34]. A variety of names, including osteogenic stem cells, marrow stromal fibroblastic cells [35], BMSCs [36], MSCs [37], stromal precursor cells and, more

recently, skeletal stem cells [38] have been ascribed to this population of cells although, currently, MSCs (undifferentiated multipotent cells of the mesenchyme) appears to be the favored term and will be used in this review [35–38]. MSCs give rise to a hierarchy of cell populations within bone to give, in essence, a developmental continuum that can be artificially divided into a number of developmental stages, including MSC, determined osteoprogenitor cell, preosteoblast, osteoblast and, ultimately, osteocyte. A number of criteria need to be fulfilled before a cell can be termed a stem cell, namely, self-renewal, the ability to differentiate into more than one cell type and the capacity for cell division to be maintained throughout life. It is important to note therefore that osteogenic progenitors are an intermediate between a stem cell and differentiated progeny (i.e., osteoblast) and significantly, to date, a homogenous human MSC population has yet to be isolated.

The ongoing progress of stem cell biology has led to its interest in many clinical applications. However, in orthopedics, the use of bone marrow to augment allograft is not a new practice. HBMCs have been isolated from bone marrow and injected percutaneously for the treatment of tibial nonunions [39] and avascular necrosis [40] of the hip with reported success. Outside the clinical domain, a number of animal studies have demonstrated that tissue engineering techniques using autologous MSCs seeded onto a scaffold lead to bone regeneration at a defect site [41–44]. However, until recently little work had investigated the potential role that MSCs could play in IBG.

For MSCs to play a role in IBG it is necessary to first determine if, when combined with allograft, these cells could survive the impaction process. A study by Mushipe has confirmed that a human osteosarcoma cell line (MG63 cells) seeded onto morselized bovine allograft can survive a limited number of impactions in an acetabular impaction grafting model [45]. In brief, using morselized cancellous bone graft as a porous scaffold, MG63 cells were seeded on the scaffold and impacted into an acetabulum cup model using a mechanical device constructed from data obtained during impaction grafting by an orthopedic surgeon. Immediately after impaction, cells were trypsinized from the scaffold and processed for cell survival rates using the double-stranded DNA PicoGreen® assay. Significant reductions in viable cells were observed between the fifth impact and both the first and second impacts ($p < 0.01$ and $p < 0.05$, respectively). Cell survival rate was 21.5% after five impacts. It is important to note the IBG technique is highly operator-dependent and the forces that are employed to the graft will vary considerably between surgeons. Therefore, it is critical to establish a range of forces within which the cells are able to survive. A similar concept was tested by Korda and colleagues, who confirmed the viability of sheep mononuclear cells seeded onto allograft after impaction with a range of forces [46]. Sheep MSCs were isolated and culture expanded under basal conditions. Cells were seeded, agitated for 2.5 h and left in culture for a further 72 h prior to impaction. The cell–graft composites underwent various impaction forces (0, 3, 6 and 9 kN). These were demonstrated to represent the range of forces encountered by different surgeons performing femoral IBG. The impaction force was regulated by the drop height of a 1 kg weight. Almar blue assay was used to determine and quantify cell viability. The results demonstrated that the cells remained viable in 3 and 6 kN groups (as evidenced by increased percentage of absorbance of Almar blue) but were reduced after 9 kN impaction force. The authors concluded that the addition of MSC to allograft could survive normal impaction forces in revision THR, but recommended avoiding excessively high impaction forces.

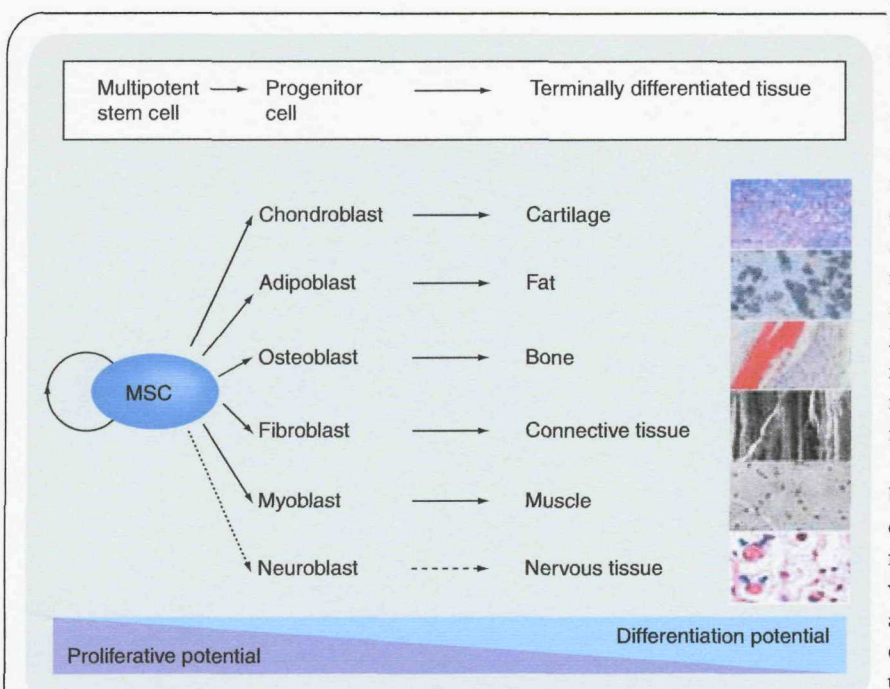


Figure 1. The differentiation of MSCs from a self-renewing MSC to generate all mesenchymal lineages. Note the postulated steps are highly schematic (the process is a continuum) and the potential for neuronal tissue formation remains controversial and is indicated by the dashed line.
MSC: Mesenchymal stem cell.

Initial viability studies using human bone marrow stromal fractions seeded onto human bone allograft were performed by Tilley and colleagues working within the Bone and Joint Research group at Southampton, UK [47]. Preliminary experiments involved the seeding of culture-expanded bone marrow mononuclear cells onto highly washed allograft. The resulting living composite was then impacted prior to incubation *in vitro*. The impactor was designed using data from

previous force plate analysis studies performed by Brewster and colleagues (FIGURE 2) [48]. Each sample received 72 impactions at a frequency of 1 Hz in the manner described by Dunlop and colleagues [49], which transmitted a total energy equivalent to that imparted during a standard femoral impaction.

The study demonstrated cellular proliferation and increasing osteoblastic activity between 7 and 17 days of culture. Confirmation of viability prompted a parallel *in vitro* and *in vivo* subcutaneous nude mouse model over a 28-day period. Allograft samples seeded with culture-expanded HBMSC grown under osteogenic conditions and implanted *in vivo* demonstrated enhanced osteoid production when compared with the current gold standard of impacted allograft alone. Further studies compared the immunoselected (STRO-1$^+$) MSC fraction and unselected HBMSCs were subsequently performed both *in vitro* and *in vivo*. The monoclonal STRO-1 immunoglobulin M antibody was used to isolate the CFU-F in adult bone marrow [50]. STRO-1 recognizes a trypsin-resistant cell surface antigen present on a subpopulation of human bone marrow-derived colony forming cells [51] and has been used to demonstrate that a subpopulation isolated using STRO-1$^+$ from human bone marrow cells is capable of osteogenic differentiation [52]. Enhanced survival of the STRO-1$^+$ selected cells following impaction compared with unselected HBMSC was observed *in vitro* and *in vivo* after 28 days with evidence of increased proliferation on the prepared allograft. This composite would appear to be the cell fraction of choice in the clinical setting, although the processing time to generate such fractions (compared with isolating unselected fractions) and the marked donor-dependent expression of STRO-1 [53] may preclude this approach at present. In addition, the higher cell division rates seen in enriched MSC populations following immunoselection with STRO-1$^+$ selected, as opposed to the unselected, fractions [54] may prove detrimental to the graft in the short term by encouraging early graft resorption, potentially destabilizing any prosthesis that it was supporting. A similar phenomenon was encountered with the use of OP-1 (BMP-7) in a femoral impaction grafting sheep model [25].

The earlier success with confirmation of cell viability after impaction led to further studies performed by Bolland and colleagues examining the potential mechanical effect that culture-expanded human bone marrow stromal fractions seeded onto allograft could provide in contrast to allograft alone [55].

A similar *in vitro* study was performed with HBMSC culture expanded under osteogenic conditions, seeded onto highly washed morselized allograft and impacted with the equivalent forces of a standard femoral impaction. The mechanical properties of the graft were determined by measuring the shear strength and its constituent components, interlocking particle cohesion (c) and friction angle, using a Cam shear tester at 2- and 4-week time intervals (FIGURE 2). This device enables the behavior of particulate materials in combined normal and shear loading to be characterized. At 2 and 4 weeks, good cell viability was observed in both the center and periphery of all samples (FIGURE 3, 4-week data shown). There was an increased number of cells staining, as observed using the cell tracker green probe within the 4-week samples. DNA levels were significantly higher in the HBMSC samples over the controls at 2 weeks and continued to increase in the 4-week samples, with a statistically significant difference. Interestingly, specific alkaline phosphatase levels originally rose in the HBMSC group at 2 weeks but subsequently fell by 4 weeks. These findings were in keeping with work performed by Pockwinse and colleagues who investigated the levels of a variety of markers of bony expression over time on rat calvarial cells [56]. Pockwinse demonstrated peak alkaline phosphatase activity at day 14–17, with a subsequent fall off. Positive staining for type I collagen and Alcian blue/Sirius red further confirmed osteogenic differentiation of the seeded HBMSC population (FIGURE 3B & C).

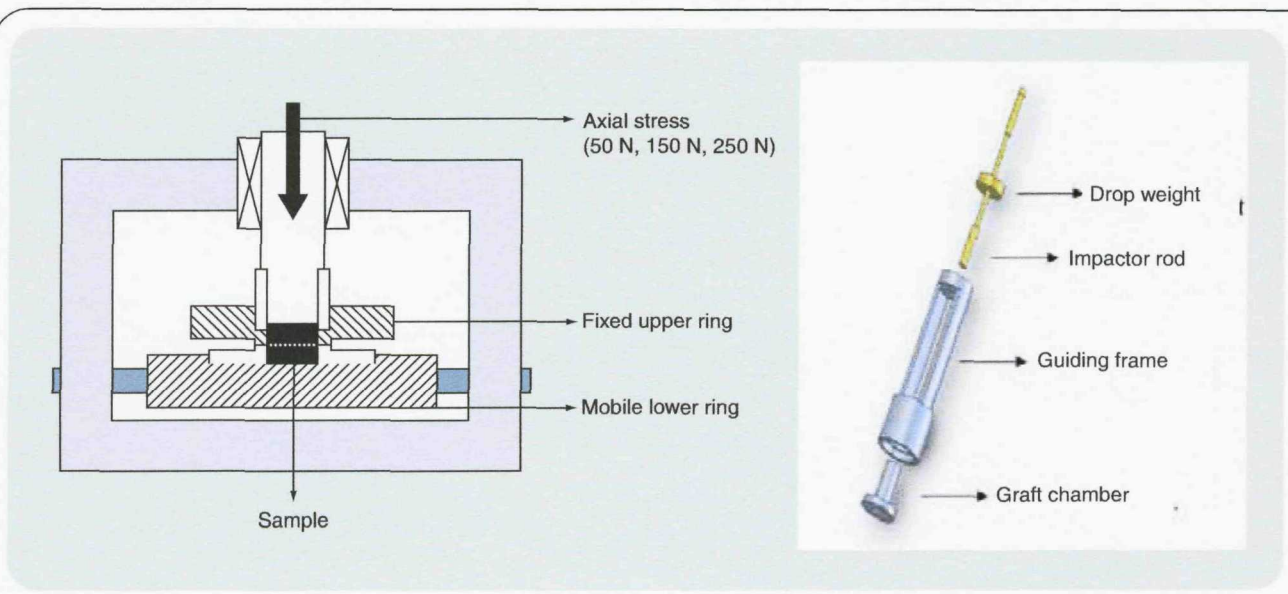


Figure 2. Impactor design and Cam shear tester.

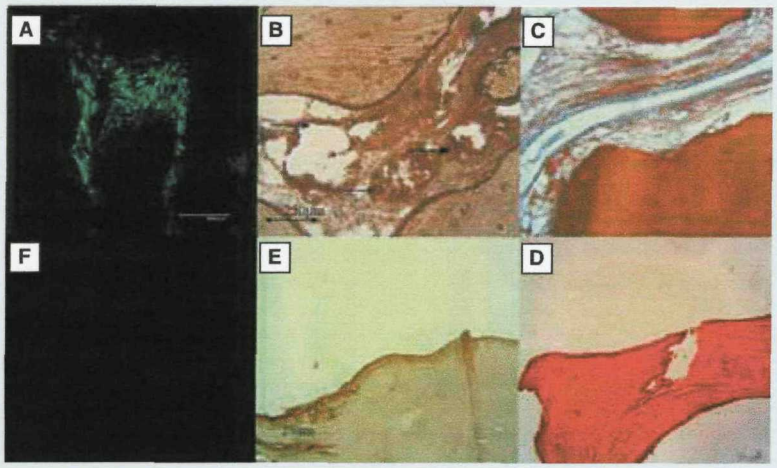


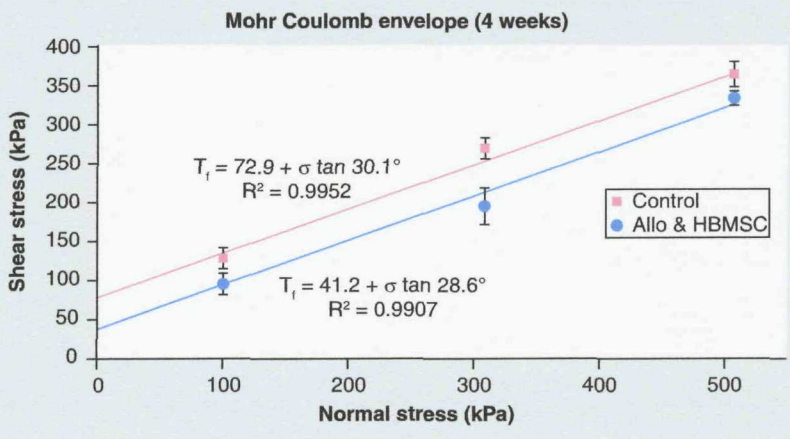
Figure 3. Ethidiumhomodimer-1 and cell tracker green, collagen type I and Alcian blue/Sirius red staining in human bone marrow stromal cell/allograft composites (A,B,C) and allograft alone (D,E,F).

Mechanical shear testing demonstrated an increase in interparticulate cohesion and shear strength at all compressive stresses at 2 and 4 weeks in the HBMSC group but with no additional rise between 2 and 4 weeks (FIGURE 4). At 4 weeks the internal friction angle was observed to rise. We have postulated that the addition of cells has a twofold action: initially to increase the cohesion between individual particles (resulting in an increase in c) and, second, through *coating the graft and proliferating*, the volume of the particles increases. Both these factors make it more difficult for the particles to slide across each other and 'shear' (resulting in an increase in Φ) and, therefore, cause an increase in shear strength with the clinical implications therein; ↑c + stan↑F = ↑t.

Mechanical stability is essential to allow early weight bearing, which is important in loading and stimulation of the graft, with possible faster graft incorporation [57]. The increased shear strength provided by an allograft–HBMSC composite could allow not only early mobilization of patients but also, significantly, lead to improved graft incorporation.

Although these results are encouraging, caution must be advocated in advance of validation of mechanical enhancement in an *in vivo* model. The balance between bone resorption and bone regeneration normally seen in fracture healing and graft incorporation may be altered when the living composite is subjected to loading and a response from the host's own cells. This will need to be established to ensure that the augmentation of allograft with HBMSCs does not result in accelerated bone resorption prior to bone regeneration, with consequent aseptic loosening and implant failure.

These studies suggest that HBMSCs could play an important role in improving both the biological and mechanical properties of allograft. However, it is also important to note that allograft supplies are limited. This, along with the advances in tissue engineering, has led to the interest in synthetic scaffolds. Furthermore, the potential immunogenic reaction that can arise with the use of allograft would be mitigated with appropriate synthetic scaffolds. A living composite of synthetic graft seeded with HBMSC, which could survive the impaction process, differentiate and proliferate along the osteogenic lineage with similar or even greater mechanical properties, is an attractive proposition and could be used in combination with, or as a replacement for, allograft. The earlier success of impacted allograft seeded with HBMSCs, along with the ever apparent clinical need for alternatives to allograft, has led us to a series of proof-of-concept studies with the synthetic graft, PLA, (FIGURE 5A). PLA is a degradable polymer that is US FDA



	Interlocking (kPa)	Friction angle (deg)	Shear strength (kPa)
Allo & HBMSC (n = 3)	72.9*	30.1	276*
Control (n = 3)	41.2*	28.6	232*

Figure 4. Shear strength envelopes at 4-week time intervals from allograft/HBMSCs composite and allograft alone, showing regression analysis trend lines and standard deviation error bars.
*Statistical difference; $p < 0.01$.
Allo: Allograft; HBMSC: Human bone marrow stromal cell.

approved and used widely in the formation of resorbable sutures, scaffolds and drug-delivery devices. Integral to the success of a scaffold is its biocompatibility, which is dependent upon the adherence of stem cells and progenitors to its surface. Furthermore, the ability of progenitor cells to dynamically repopulate a scaffold surface is key in the development of new tissue structures. Yang and colleagues previously demonstrated successful adhesion, spreading, growth and differentiation of human osteoprogenitor cells on surface-modified 2D PLA films and 3D PLGA (75:25) scaffolds [58]. But could this relatively inexpensive, biocompatible, nonimmunogeneic scaffold with potential for encapsulation of growth factors for slow-release delivery, play a role in IBG? Initial *in vitro* studies performed with cultured expanded HBMSCs seeded onto morselized PLA impacted (with equivalent forces of a standard femoral impaction) and cultured under osteogenic conditions indicate good cell viability after 4 weeks. *In vivo* studies were performed to establish not only new bone regeneration but also the angiogenic response of the host to the living composites [BOLLAND *ET AL.*, UNPUBLISHED DATA].

Neovascularization is paramount to the delivery of cells, factors and nutrients required in the formation of bone. Quantification of new vessel formation is primarily reliant upon histology and various protocols for counting vessel number and length. A new technique was developed using a radio-opaque contrast infiltrated into the microvasculature and subsequently visualized using micro-computed tomography (micro-CT) imaging with 3D reconstructions (FIGURE 5B). Contained samples of impacted PLA seeded with HBMSCs and PLA alone were implanted subcutaneously in nude mice for 4 weeks. Allograft–HBMSC composites and allograft alone were also impacted and implanted in an identical manner. Macroscopically there were increased numbers of vessels surrounding and penetrating the PLA or allograft–HBMSC composites compared with PLA or allograft alone (FIGURE 6B). 3D micro-CT reconstructions allowed calculation of vessel number penetration into the samples and total volume of vessels within the samples (FIGURE 5B, inset PLA alone). Again, there was a significantly greater number of vessels penetrating and a greater total vessel volume within the PLA–HBMSC composites compared with PLA-alone samples. Histological analysis confirmed good cell viability (FIGURES 5C & 6C) and evidence of new bone formation with abundant staining for type I collagen in the PLA–HBMSC composite samples (FIGURE 5E). Immunohistochemical staining for von Willebrand factor (FIGURE 5F), a marker of angiogenesis, was abundant in the PLA–HBMSC group compared with the PLA-alone group.

The connection between osteoblasts and endothelial cells has been established and is composed of both indirect (regulation of interstitial temperature by microcirculation) and direct influences (production of angiogenic factors, such as vascular endothelial growth factor [VEGF] by osteoblasts). VEGF receptor number is thought to be upregulated by exposure to hypoxic conditions (i.e., similar to those encountered during impaction). Therefore, it may be conceivable that increased angiogenesis observed in the impacted samples results from upregulation of VEGF receptors. In addition to HBMSC surviving impaction and promoting bone regeneration, the addition of HBMSC cells also appears to support angiogenesis with the successful stimulation of new bone formation and revascularization demonstrated on both allograft and synthetic graft *in vivo*.

Translation from laboratory to theater

The reconstruction of extensive areas of bone loss arises not only in revision hip and knee surgery but also as a result of tumor, infection or other pathologies. Historical studies by Burwell using an animal model demonstrated that bone allograft seeded with autogenous marrow as a composite graft resulted in considerably more new bone than either of the components of the graft transplanted separately. While this technique has yet to become standard clinical practice, autologous marrow is increasingly being used in conjunction with synthetic grafts in spinal fusion [59–61], and maxillofacial reconstructive facial surgery [62]. A number of clinical studies have evaluated the separate use of bone marrow to augment osteogenesis [39,40,63,64] but little work has been performed on its combination with morselized allograft. Along with the encouraging *in vivo* evidence of new bone formation and

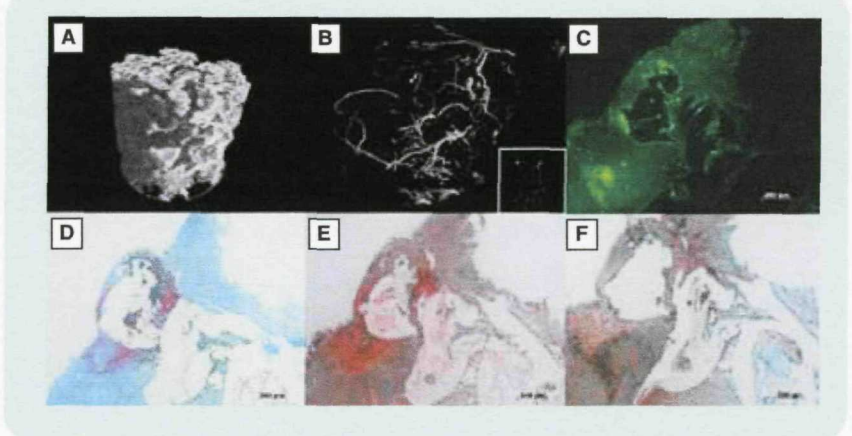


Figure 5. (A) 3D reconstruction of impacted poly(lactic acid); **(B)** Neovascularization surrounding impacted poly(lactic acid)–human bone marrow stromal cell composite, inset (poly(lactic acid) alone); **(C)** Cell viability in poly(lactic acid)–human bone marrow stromal cell composite using ethidium homodimer-1/cell tracker green immunoflourescent staining. Histological staining of poly(lactic acid)–human bone marrow stromal cell composites for **(D)** Alcian blue/Sirius red **(E)** type I collagen and **(F)** Willebrand factor demonstrating abundant positive staining for new bone and blood vessel formation.

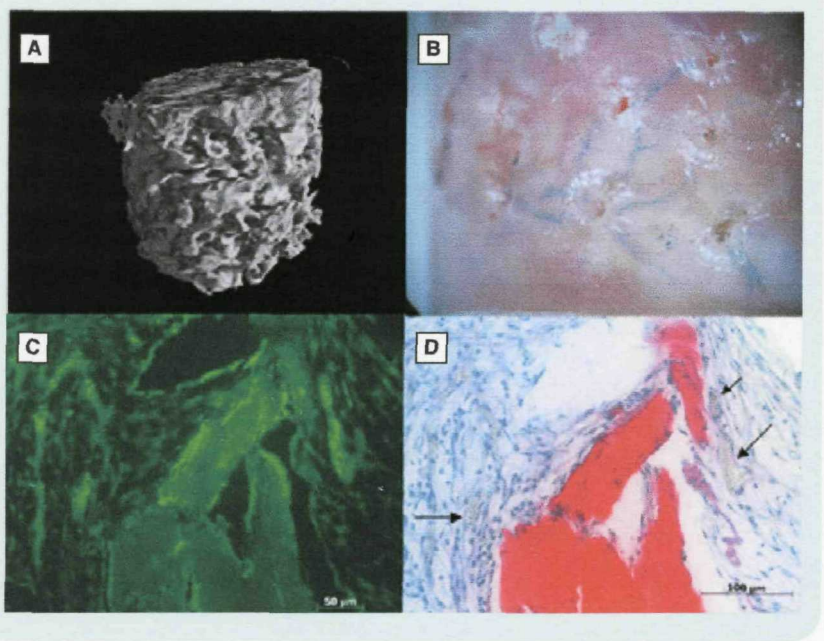


Figure 6. (A) 3D reconstruction of impacted allograft; **(B)** Penetration of blood vessels through container onto surface of impacted allograft–human bone marrow stromal cell composite; **(C)** Cell viability in allograft–human bone marrow stromal cell composite using ethidium homodimer-1/cell tracker green immunoflourescent staining. Histological staining of allograft–human bone marrow stromal cell composites for **(D)** Alcian blue/Sirius red, demonstrating a plethora of cells adherent to, and surrounding, the impacted allograft with positive staining for extracellular matrix production and evidence of new blood vessel formation (arrows).

neovascularization observed in impacted HBMSC–allograft composites, we applied principles of a tissue engineering paradigm and techniques modified from Burwell's original work from the laboratory to the operating theater [65]. Autologous HBMSCs were seeded onto washed morselized allograft and the resulting composite impacted *ex vivo* forming a solid construct with which to fill pathological defects in the proximal femur in two separate clinical cases. In addition to its space-filling ability, the osteogenic, osteoinductive and osteoconductive properties of the graft were examined.

Case 1 was a 62-year-old man who presented with a 1 year history of hip and groin pain. Radiographs revealed a large well-circumscribed focal lesion seen in the anterior portion of the femoral neck on the right side, diagnosed as representing a benign nonossifying fibroma (FIGURE 7A & B). Owing to the anatomical site and the patient's symptoms, there was a high risk of a fracture occurring through the lesion.

Case 2 was a 39-year-old man who presented with a 6-month history of groin pain. A total of 18 months earlier he had sustained an intracapsular neck of the femur fracture that was treated with internal fixation. Radiographs demonstrated advanced avascular necrosis of the femoral head (FIGURE 7C & D). Owing to the intrusive nature of the symptoms and the radiological evidence of avascular necrosis, it was decided to remove the metal work and bone graft from the femoral head and neck.

Marrow was aspirated from the posterior superior iliac crest and seeded onto a prepared, washed morselized allograft. The seeded graft was left for 40 min to allow adherence of the marrow-derived osteoprogenitor cells prior to impaction into the defect. Samples of the impacted graft were taken for *in vitro* analysis of cell viability, histology and biochemical analysis of cell number and osteogenic enzyme activity.

Both patients made a rapid clinical recovery after an overnight stay. Imaging confirmed filling of the defects with increased density on plain radiographs suggesting good impaction of the graft composite. Immunohistochemical staining of graft samples demonstrated that a living composite graft with osteogenic activity had been introduced into the defects as evidenced by cell tracker green viability and alkaline phosphatase expression and specific activity. Radiographs at 12 weeks (FIGURE 7E & F), and CT slices at 6 months (FIGURE 7E & F INSERTS), showed satisfactory appearance of bone incorporation. In case 2, at the 6-month follow-up, there was no progression of avascular necrosis both clinically and radiologically (FIGURE 7G & H).

This innovative impaction approach has a number of advantages over the current gold standard of using allograft or synthetic grafts for the filling of large voids:

- The procedure fulfils the triad of osteogenesis, osteoinduction and osteoconduction with immediate effect
- The procedure can be performed under a single anesthetic without the removal of tissue from the operating theater, thus avoiding issues of sterility, associated with further expansion of marrow stromal cells
- The procedure provides a rapid, cost effective, facile approach, applicable to a number of clinical orthopedic scenarios

Expert commentary

With the increasing knowledge, versatility and adaptability of MSC populations, the use of tissue engineering principles to augment present day orthopedic treatment modalities augers well for the future.

This review has concentrated on the field of IBG as a treatment technique to restore bone loss during revision hip surgery but there are multiple areas in orthopedic practice when the surgeon is faced with the difficult scenario of filling large bone defects, for example, following trauma or as a result of resection for infection or tumor. Thus, these principles can be modified and adjusted for a number of clinical situations.

Research into IBG has fundamentally looked at improving the mechanical rather than the biological properties of the graft, but this is now being reversed with our emergent knowledge of MSCs and BMSCs. These studies indicate there is emerging *in vivo* and *in vitro* evidence that HBMSCs seeded onto allograft or synthetic graft (PLA) can withstand the forces of a standard femoral impaction and differentiate and proliferate along the osteogenic lineage. The living composites thus formed also appear to induce mechanical enhancement, as well as encouraging neovascularization. However, these are only short-term studies. Biological, as well as mechanical, factors control the complex process of bone remodeling with a delicate balance between bone resorption and bone regeneration. With the impacted graft in IBG playing such an important mechanical role it will be important to ensure that the mechanical properties of the graft, either bone or absorbable synthetic graft, are not temporarily reduced by a biological stimulus that disturbs the bone remodeling equilibrium. Strategies to aid these issues include the addition of nonabsorbable components, such as HA, either to bone or synthetic graft, creating an attractive alternative to augment and improve the control over the potential changing mechanical properties of the graft with time.

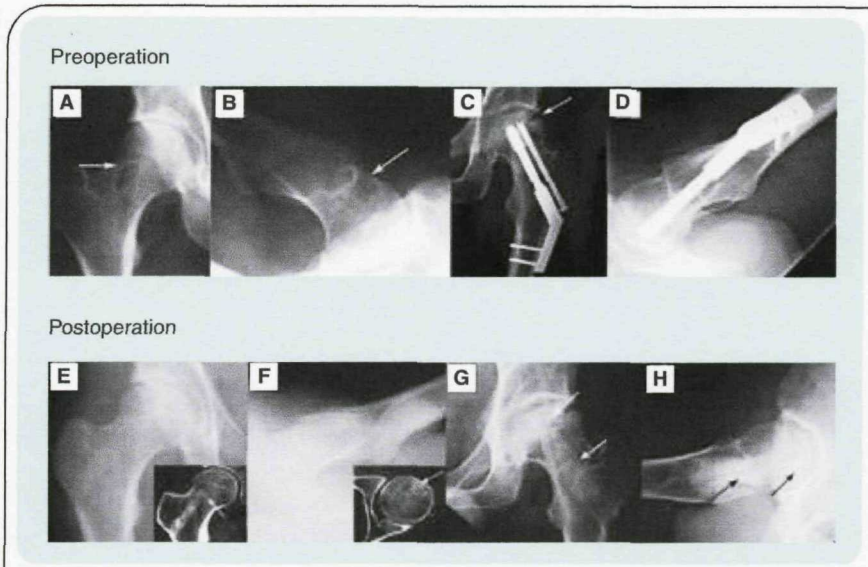


Figure 7. (A,B) Anterior/posterior and lateral hip radiographs demonstrating well-defined cystic lesion in anterior portion of the femoral neck (Case 1), and **(C,D)** severe avascular necrosis with collapse of the femoral head and areas of lucency (Case 2). Postoperative anterior/posterior and lateral radiographs of case 1 **(E,F)**. These films, taken at 12 weeks, are supplemented by computer tomography slices; coronal **(inset E)** and axial **(inset F)** at 6 months post-operatively. Radiographically the lesion has been replaced by bone at a higher density than the surrounding cancellous bone. This feature is also seen in radiographs of case 2 **(G & H)** at 12 weeks.

Despite the vast variety and developing technology in tissue engineering, a synthetic scaffold that can match the properties of allograft remains elusive. Although the fundamental question of cell survival in IBG has been resolved, there are a number of variables relating to both the scaffold and the cell selection that need to be addressed to produce the optimum living composite. In particular, the production of synthetic grafts with encapsulated growth and angiogenic factors that could result in sustained and controlled release over time providing greater control of the bone remodeling equilibrium. A key area for further investigation and refinement surrounds the ideal cell population isolation and expansion of that population. The enriched STRO-1$^+$ population with its known osteogenic potential would appear to be a useful cell fraction to exploit. However, as yet we are unaware of the effects on bone remodeling with time when introducing a composite with an enhanced osteogenic capacity. As the graft used in IBG plays such an important mechanical role when initially implanted it is paramount that the initial stability achieved through surgical impaction is not compromised by the stimulation of early graft resorption. Therefore, further longer term *in vivo* studies are required to determine the rates of bone resorption and bone regeneration using different cellular fractions with time.

The isolation and culture expansion of cells, regardless of technique, still requires removal of the original bone marrow sample from theater for processing. Despite advances in cell culture techniques multiple treatment steps will result in concerns over contamination. One possible solution would be to utilize bioreactors to control the microenvironment that is most advantageous to the creation of a product, that is, cultured expanded HBMSC populations. The three types of bioreactors that exist are batch, fed batch and continuous. A continuous bioreactor adds nutrients and removes waste products over the entire course of the reaction. The cells in these reactions utilize the nutrients present from the inflow and secrete products to the outflow where they are filtered and removed from the fluid. The development of these bioreactors along with the ever evolving 3D scaffold technology will play an important part in improving the role of mesenchymal populations in IBG.

Five-year view

There is encouraging evidence from both *in vivo* and *in vitro* studies that HBMSCs can survive the forces equivalent of a standard femoral impaction when seeded onto both allograft and synthetic graft. Further studies are required to optimize the numerous potential variables involved in utilizing HBMSCs in IBG. Areas of particular interest include the potential use of enriched osteoprogenitor populations, scaffolds encompassing multiple growth factors and development of synthetic grafts and bioreactors to create a one-step living composite for clinical use. The current drive in tissue engineering and regenerative medicine auger well for the development of platform technologies to harness the potential of mesenchymal populations in IBG and the development of

composites that provide enhanced biological, mechanical and, hopefully, improved clinical outcome for patients requiring revision hip surgery. Thus, ultimately, clinical trials will be required to establish the true potential of mesenchymal populations for use in IBG and validation of this paradigm shift with the implications therein for an increasingly aging population.

Key issues

- Each year in the UK there are over 50,000 primary hip replacement operations at a cost of £250 million. This is set to rise to 65,000 by 2026, of which 30–50% will require subsequent revision surgery.
- *Impaction bone grafting is a recognized technique to deal with loss of bone stock often encountered in revision hip surgery.* The use of morselized allograft is the current gold standard, providing mechanical support, but it offers little osteoinductive biological potential.
- Mesenchymal populations containing stem cells exist postnatally, are multipotent and have the ability to give rise to stromal lineages (bone, cartilage, muscle, tendon, ligament and fat). Bone marrow is a reliable source of human bone marrow stromal cell (HBMSC) populations.
- HBMSC, when combined with either allograft or synthetic graft (poly(lactic acid) [PLA]), can survive the forces of a standard impaction bone grafting and under osteogenic conditions, differentiate and proliferate along the osteogenic lineage. HBMSC and allograft composite confer an additional biomechanical advantage over the allograft alone.
- *In vitro* and *in vivo* impaction studies on allograft using a selected stromal cell population with enriched osteogenic potential (STRO-1$^+$) demonstrated increased cell survival over unselected stromal population (HBMSC).
- Increased neovascularization has been demonstrated *in vivo* in allograft and PLA/HBMSC composites compared with allograft or PLA alone.
- Tissue engineering principles combining morselized allograft and HBMSC composites have been utilized to fill bony voids in two clinical cases, with, to date, good clinical outcome.
- With dwindling allograft stocks, future development and studies with encapsulated release system scaffolds are essential.
- With the potential to translate this tissue engineering paradigm into the clinical domain, bioreactors will become integral to minimize the potential for contamination and produce a safe, reliable microenvironment for the production of 3D living composites.

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Taking tissue-engineering principles into theater: augmentation of impacted allograft with human bone marrow stromal cells

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Human bone marrow contains bone progenitor cells that arise from multipotent mesenchymal stem cells. Seeding bone progenitor cells onto a scaffold can produce a 3D living composite with significant mechanical and biological potential. This article details laboratory and clinical findings from two clinical cases, where different proximal femoral conditions were treated using impacted allograft augmented with marrow-derived autogenous progenitor cells. Autologous bone marrow was seeded onto highly washed morselized allograft and impacted. Samples of the impacted graft were also taken for *ex vivo* analysis. Both patients made an uncomplicated clinical recovery. Imaging confirmed defect filling with encouraging initial graft incorporation. Histochemical and alkaline phosphatase staining demonstrated that a live composite graft with osteogenic activity had been introduced into the defects. These studies demonstrate that marrow-derived cells can adhere to highly washed morselized allograft, survive the impaction process and proliferate with an osteoblastic phenotype, thus creating a living composite.

Currently, the demand for bone graft far exceeds supply [1]. This is particularly relevant in the fields of revision hip, spinal, tumor and trauma surgery. To date, autograft remains the clinical material of choice owing to its osteoconductive structural properties in addition to the innate presence of osteoinductive growth factors and osteogenic progenitor cells intrinsic to healing [1,2]. However, volumes of autograft available are limited and the additional procedure is associated with increased surgical time and donor-site morbidity, including donor site pain, bleeding and infection. In clinical situations requiring larger voids to be filled, banked fresh frozen allograft is used commonly. However, allograft is a less efficient osteoinductive agent than autograft, which directly forms bone when transplanted to a heterotopic site [3]. Although efficacious in the field of impaction bone grafting of the hip [4], allograft, by the very nature of the preparation process, remains nonviable and, thus, does not contribute to the osteogenic process directly [5]. The new bone formed from the use of allograft alone is derived from skeletal tissues of the patient at the site of the implantation.

The tremendous capacity of bone to regenerate is indicative of the presence of stem cells with the capability to self renew and give rise to daughter cells. These progenitors, termed mesenchymal stem cells or bone marrow stromal cells, exist postnatally, are multipotent and have the ability to give rise to the stromal lineages (bone, cartilage, muscle, tendon, ligament and fat) [6]. With an increasing aging population, clinical imperatives to augment skeletal tissue loss have brought these cells to the fore in combination with the emerging discipline of tissue engineering [7,8]. Historical studies by Burwell using an animal model, demonstrated that bone allograft seeded with autogenous marrow as a composite graft resulted in considerably more new bone than either of the components of the graft transplanted separately [5]. While this technique has yet to become standard clinical practice, autologous marrow is increasingly being used in conjunction with synthetic grafts in spinal fusion [9,10], and maxillofacial reconstructive facial surgery [11]. A number of clinical studies have evaluated the separate use of bone marrow to augment osteogenesis [12–15], although little work has been performed on its combination with morselized allograft. The aim of these two cases was to apply the principles of a tissue-engineering paradigm and translate techniques modified from Burwell's original work from the laboratory to the operating theatre. Marrow-derived stromal cells, which include osteoprogenitor cells, were seeded onto washed morselized allograft and the resulting composite impacted *ex vivo* forming a solid construct with which to fill pathological defects in the proximal femur. In addition to its space-filling ability, the osteogenic, osteoinductive and osteoconductive properties of the graft was examined.

Keywords: bone marrow aspirate, human allograft, impaction bone grafting

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Case one

Introduction

Benign tumors are commonly found in the proximal femur [16]. Clinical presentation is variable depending on the pathology, site and size of the lesion. The spectrum of presentation varies from asymptomatic incidental x-ray finding to those with acute proximal femoral fracture. However, most present with pain, limp and leg-length inequality [17].

Qualitatively, indications for surgery include progressive lesions causing pain or deformity and, quantitatively, if over 2.5 cm or involving at least 50% of the cortex where there is a significant risk of fracture [18,19]. Principles of prophylactic treatment remain contentious and may involve curettage of the lesion, void filling, either with bone graft or marrow injection, and mechanical stabilization. The process of reconstitution of the defect is often difficult owing to the degree of bone loss. In addition, reconstruction may be associated with significant blood loss, donor-site morbidity, infection, restriction of activity and recurrence [20].

Case history

A male aged 62 years presented with a 1-year history of right hip pain. The patient described a dull ache around the lateral trochanter, passing down the anterior thigh with significant deterioration in the months leading up to the procedure. The patient reported difficulty in walking and was beginning to experience night pain, although otherwise the patient was systemically well with no significant past medical history. On examination he walked with an antalgic gait. There were no other positive findings and no restriction of hip movements.

Plain radiographs (Figure 1A & B) showed a well circumscribed focal lesion in the anterior portion of the femoral neck on the right side. The lesion was expansile with slight distortion of the anterior femoral neck cortex (though this remained intact) and diagnosed as representing a benign nonossifying fibroma.

Exacerbation of the patient's pain and proximity of the lesion to the anterior cortex indicated the potential of impending fracture through the lesion. Surgical intervention was recommended with appropriate counseling, including the risks of intraoperative femoral neck fracture.

Surgical technique

Morselized allograft was prepared from a banked fresh frozen femoral head. After denuding the femoral head of articular cartilage, fibrous tissue, osteophyte, cystic areas and calcar, the remaining bone was milled using the large and small cylinders of a Noviomagus bone mill. This combination of mill sizes ensured optimal particle size distribution (grading) most resistant to shear strength [21]. The morselized aggregate was serially washed with pulsed lavage and 6% hydrogen peroxide until no further fat or blood was visible. Finally, the graft was washed with normal saline and mixed with 500 mg vancomycin powder.

Bone marrow aspiration was performed with the patient in the lateral position in the operating theatre under strict aseptic conditions. A single incision was made over the posterior superior iliac spine and multiple passes were made with a trephine to prevent dilution of the aspirate with hemopoietic cells (Figure 2A). A total aspirate volume of 36 ml was obtained, 2 ml was retained for *ex vivo* analysis and the remainder immediately seeded onto the graft and covered.

The patient was then positioned supine on the operating table with standard lateral preparation and draping. Image intensification was used to determine the incision point. A 1.5 cm incision was made in the skin over the lateral thigh, followed by sharp dissection to bone. A guide Kirschner wire was introduced through the lateral cortex and, under image intensification, directed towards and through the cystic lesion. A 10 mm cannulated femoral canal corer (Stryker Int. IBG kit) was passed over the Kirschner wire (Figure 2B), removing a tunnel that included the contents of the cyst, which was used for histological analysis. Aggressive curettage using a serrated spoon was performed to the cyst walls under image intensification.

Graft was introduced into the corer (Figure 2C) and impacted *ex vivo* to produce a solid core. This was extruded with further impaction in a retrograde fashion into the tunnel defect under image-intensified control. This process was repeated with grafting to the lateral femoral cortex. The wound was closed following hemostasis and washout.

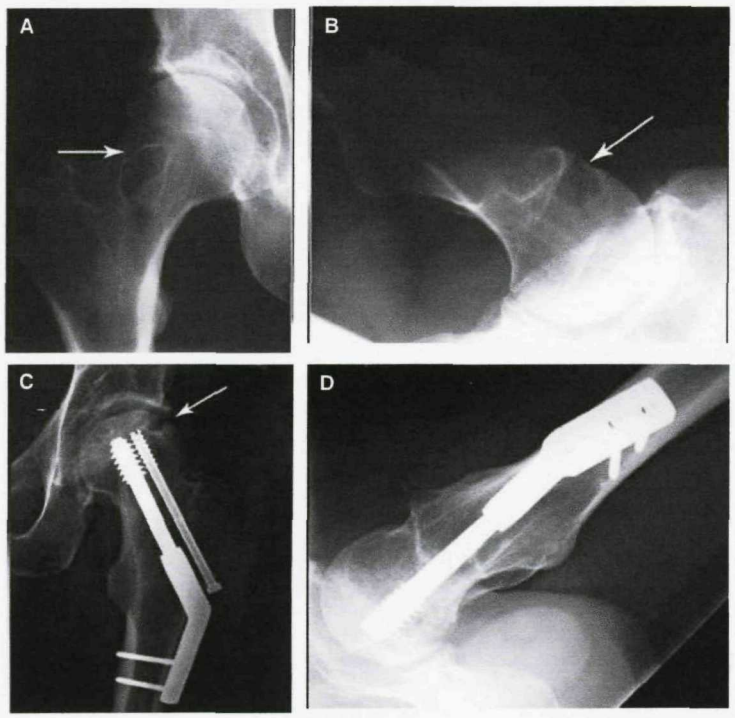
Representative samples, including bone marrow aspirate, impacted allograft seeded with bone marrow stromal cells and allograft alone, were taken for biochemical and histological analysis.

Case two

Introduction

Avascular necrosis (AVN; temporary or permenant cessation of blood supply to bone) of the femoral head is a potentially devastating complication of intracapsular femoral neck fracture

Figure 1. Pre-operative radiographs (A & B: case one; C,D: case two).



Preoperative anterior–posterior **(A)** and lateral **(B)** radiographs of the right hip in case one. The lesion is seen residing in the anterior third of the femoral neck, distorting the anterior cortex. Anterior–posterior **(C)** and lateral **(D)** of left hip in case two. There is evidence of destructive avascular necrosis seen superiorly and laterally in the femoral head.

in young patients. Surgical options for early disease (Ficat Stage I and II) [22] include core decompression and, more recently, implantation of autologous bone marrow cells [12,23,24]. Salvage procedures for advanced disease, including the use of vascularized fibular grafts [25], are technically demanding. Alternatives include the Bonfiglio strut graft (nonvascularized), proximal femoral osteotomy to alter the pattern of stress transfer and vascularized pedicle flaps. Each procedure has substantial morbidity and, to date, there are no studies indicating the optimal procedure in the treatment of advanced disease. We present the first reported combination of these procedures, utilizing the positive attributes of each: decompression, osteoinduction/conduction and structural support.

Case history

A male aged 39 years presented with a 6-month history of pain and stiffness in his left groin and buttock. 18 months earlier he had fallen off his bicycle, sustaining an intracapsular fracture of his left femoral neck. He was treated within 12 h with a two-hole dynamic hip screw (DHS) and derotation screw and made an uncomplicated postoperative recovery, remaining pain free for 1 year. The resultant pain was mechanical in nature and did not disturb him at night. On examination, there was no obvious muscle wasting and Trendelenberg test was negative. There was no bony tenderness, although internal rotation was reduced on the left.

Pelvis and left hip radiographs showed two defined areas of lucency in the left femoral head with apparent disruption of the articular surface and a degree of collapse of the femoral head (Figure 1C & D).

Owing to the intrusive nature of the symptoms and the radiological evidence of avascular necrosis, it was decided to remove the metalwork and bone graft the femoral head and neck. Morselized allograft, seeded with autologous marrow stromal cells, was impacted into the deficient area.

Surgical technique

A similar procedure as in case one was performed. The patient was placed in the lateral position, prepared and draped. 50 ml of bone marrow was aspirated from the posterior superior iliac spine and seeded onto prepared highly washed morselized allograft obtained from one donated fresh frozen femoral head. The previous incision was used to expose and remove the dynamic hip screw and derotation screw. The screw tracks were curetted and, under image intensification, the sclerotic bed was drilled. The seeded allograft was then impacted into the femoral neck using the 10-mm cannulated femoral corer, as before, without disruption to the articular cartilage. Samples were taken for biochemical and histological analysis.

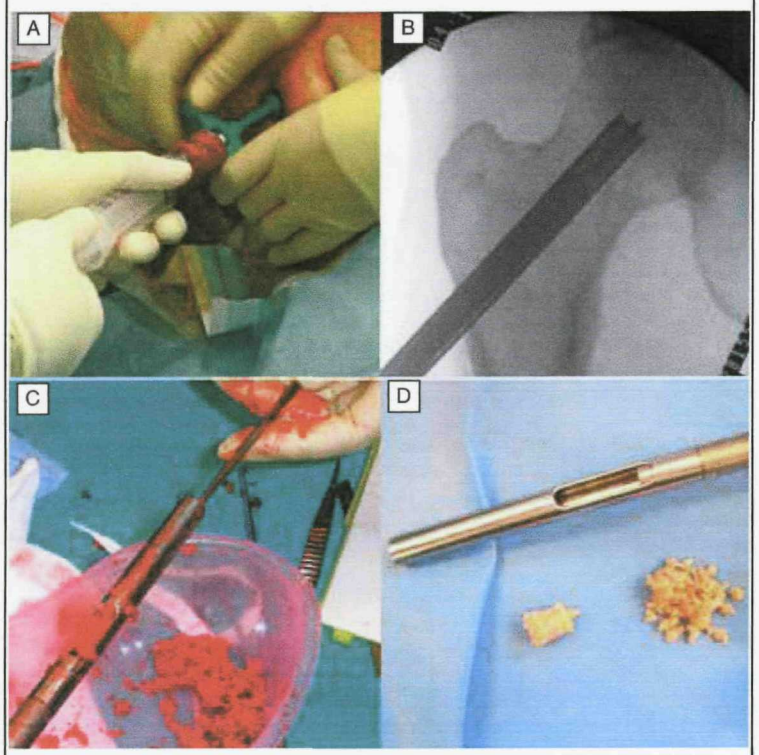
Materials & methods

Biochemical technique

Assay of alkaline phosphatase-positive colony-forming units

Bone marrow aspirate (2 ml) taken intraoperatively was immediately plated, in theatre, into tissue culture flasks (75 cm^3, n = 3). 8 ml of media (minimum essential medium: α-modification, 10% fetal calf serum, 100 µM ascorbate-2-phosphate and 10 nM dexamethasone) was added to each flask. The plates were incubated at 37°C in 5% CO_2. The media was changed, including two washes with phosphate buffered solution (PBS) on the seventh day. On day 9, the samples were

Figure 2. Surgical technique.



(A) Bone marrow aspirate taken from posterior superior iliac spine. **(B)** Intraoperative image of bone corer being railroaded over guide Kirschner-wire. **(C)** Seeded graft introduced into the corer and impacted *ex vivo*. **(D)** *In vitro* laboratory simulation of graft impaction (graft pre- and post-impaction).

stained *in situ* for alkaline phosphatase activity. The number of alkaline phosphatase-positive colony forming units-fibroblastic (CFU-F; a colony is defined as containing more than 32 cells), were counted in each flask.

DNA & alkaline phosphatase-specific activity

Samples of impacted seeded allograft were taken in theatre and transferred to two, six-well plates and the media added. The plates were incubated at 37°C in 5% CO_2 and the media was changed every 4 days. Control samples of highly washed morselized allograft were also cultured under identical conditions. At 1 week, constructs were washed with PBS, then incubated with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%) at 37°C and 5% CO_2 interspersed twice with vigorous vortexing. Cells were collected by centrifugation (13,000 rpm for 10 min at 4°C) and then resuspended in 1 ml 0.05% triton X-100. Lysis was achieved by freezing and thawing, and samples were stored at -20°C until assayed. Lysate was measured for alkaline phosphatase activity using p-nitrophenyl phosphate as a substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3 at 25°C; Sigma; Poole, UK). DNA content was measured using PicoGreen®, according to manufacturer's instructions (Molecular Probes; Paisley, UK). Alkaline phosphatase-specific activity was expressed as nanomoles of p-nitrophenyl phosphate/h/ng DNA.

Measurement of graft compaction

A load cell of 5 kN capacity taken from an Instron 1173 materials testing machine (Instron Ltd.; High Wycombe, UK) was used in the laboratory to measure the forces imparted to the morselized graft during graft compaction. The 10-mm bone corer was loaded with representative amounts of wet washed allograft and the graft compacted by repeated blows to the plunger in the same fashion as in theater (Figure 2D). For each graft sample, three groups of ten blows were applied to the plunger and the peak and mean forces recorded. The mean and standard deviation of the force delivered per blow for three different surgeons are shown in Figure 3.

Results

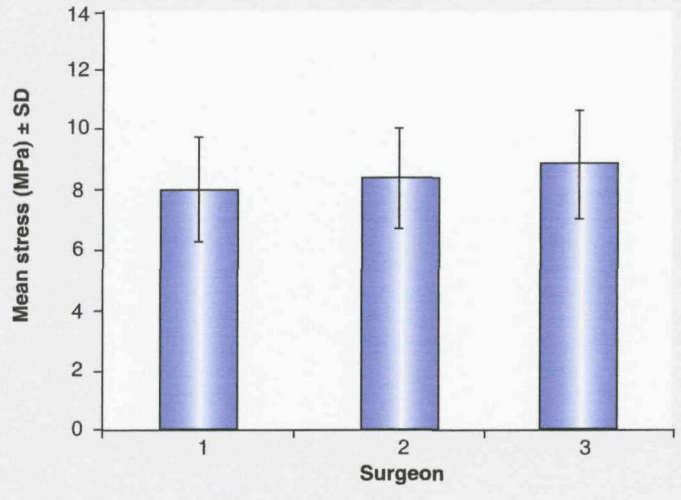
Clinical & radiological

Both patients left hospital on day 1 postoperatively with simple analgesics and mobilized with protective weight bearing for 6 weeks with two crutches, whereupon both patients were reviewed and were found to be asymptomatic. Radiographs at 12 weeks (Figure 4A & B) and computerized tomography slices at 6 months (Figure 4A & B, inserts) showed satisfactory appearances of bone incorporation. In case two, at 6 months follow-up, there was no progression of AVN both clinically and radiologically (Figure 4C & D).

Impaction forces

The mean of the peak force delivered per strike to the graft was 0.7 ± 0.13 kN (standard deviation [SD]), corresponding to average peak stresses within the graft of 8.3 ± 1.5 MPa (SD). There was no statistical difference between the three operators (Figure 3). This stress was greater in magnitude to our estimated peak stresses imparted in a standard femoral impaction bone grafting in revision hip surgery (0.8 MPa). This was an expected finding, since the pellet of allograft produced from the apple corer in the theatre and laboratory appeared more compact than impacted graft from a femoral impaction bone grafting.

Figure 3. Mean peak stresses per strike imparted to the bone graft from three surgeons.



SD: Standard deviation.

Alkaline phosphatase-positive CFU-F & enzyme activity

The bone marrow aspirate from case one yielded 4×10^5 nucleated cells/ml and 2.75×10^6/ml was yielded from the patient in case two. The average number of alkaline phosphatase-positive CFU-F formed was 2.18 CFU-F/10^6 nucleated cells (range: 1.64–2.54) (Figure 5A). The presence of large numbers of erythrocytes, in the absence of fractionation in theatre, resulted in a reduced surface area and suboptimal conditions for adherence and proliferation of the nucleated stromal cell population with a lower CFU-F yield. The exact mechanisms of reduced proliferation are not currently understood, although expansion in the presence of high erythrocyte number appears a factor.

Biochemical

Samples from both cases of allograft seeded with bone marrow demonstrated increased DNA content and specific alkaline phoshatase activity (case one, day 0: 0.05 nM pNPP/h/ngDNA; case two, day 7: 23.7 nM pNPP/h/ngDNA) when compared with allograft alone (0 nM pNPP/h/ngDNA).

Histological

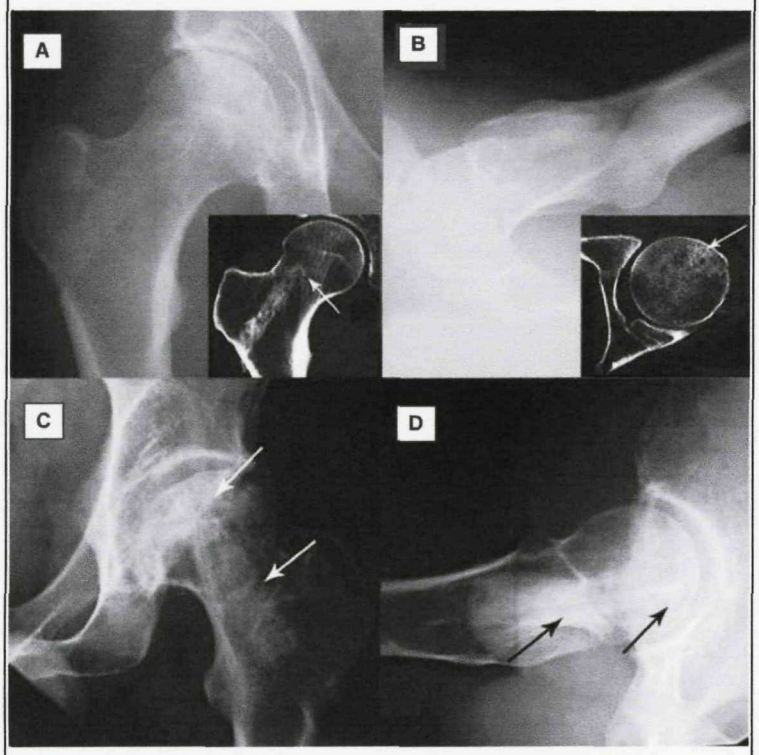
The histological analysis of the sample of tissue containing the cyst from theatre showed macroscopic and microscopic appearances consistent with a nonossifying fibroma. Immediate live/dead staining confirmed cell viability postimpaction (Figure 5C) and no cell activity from the allograft alone (Figure 5B). Repeat staining at 1 week demonstrated increased live cell numbers on the graft (Figure 5D). Staining with hemotoxylin and eosin showed dark staining marrow stromal cells adherent to the graft surface (Figure 5F). No cells were visible adherent to or surrounding the allograft alone (Figure 5E). Immunohistological staining demonstrated a proportion of the marrow stromal cells at the allograft surface and in the surrounding hematoma positive for bone sialoprotein (Figure 5H).

Discussion

This study demonstrates the potential to use autogenous human bone marrow cells for bone impaction and the clinical potential therein. We have demonstrated that autologous human bone marrow stromal cells can adhere to highly washed allograft, proliferate and express an osteogenic phenotype after impaction into a contained defect. In both cases, bone marrow aspirate alone was used, in the absence of cell selection.

Central in this impaction procedure is the correct preparation of allograft to enable seeding of marrow stromal cells. *In vitro* data demonstrate that inadequate washing reduces the adherence, survival and proliferative potential of seeded cells (Tilley *et al.*, Unpublished Data). Sequential hydrogen peroxide washing, followed by quenching in normal saline, were used to remove the majority of lipid contaminants. Banked allograft remains a potential source of retained immunogenicity (and disease transmission). However, this can be attenuated by the process of freezing the marrow elements within the cancellous bone, attributed to raising an immune response remain. In addition, removal of lipid and marrow fluid results in a stronger, compacted graft that displays greater resistance to shear [21]. Histological analysis of washed allograft samples taken from theatre confirmed negligible or no bone marrow remnants within the graft infrastructure. Soil mechanical engineering principles have shown that a carefully graded and impacted aggregate will exhibit greater load-carrying capacity than a poorly graded impacted aggregate [26,27]. Morselized allograft has been combined with bone marrow to produce a living composite [5], but provides little structural support. However, as seen in revision hip surgery, impacted bone graft can withstand significant loading. In the current studies, bone marrow stromal cells were observed not only to adhere to allograft but to withstand the forces of impaction and continue to proliferate offering significant potential in orthopedic

Figure 4. Follow-up radiological imaging at 3 months.



Postoperative anterior–posterior and lateral radiographs of case one **(A & B)**. These films taken at 12 weeks are supplemented by computer tomography slices; Coronal (**A**, insert) and Axial (**B**, insert) at 6 months postoperatively. Radiographically, the lesion has been replaced by bone at a higher density than the surrounding cancellous bone. This feature is also observed in radiographs of case two **(C & D)** at 12 weeks.

practice, particularly in the field of revision hip surgery with impaction bone grafting. Improvements on bone marrow fractionation to remove contaminating erythrocyte numbers will undoubtedly improve CFU-F yield, as observed in the laboratory.

Advantages

This innovative impaction approach has a number of advantages:

- The procedure fulfils the triad of osteogenesis, osteoinduction and osteoconduction with immediate effect;
- The procedure can be performed under a single anesthetic, without removal of tissue from the operating theatre, thus avoiding issues of sterility associated with further expansion of marrow stromal cells;
- The procedure provides a rapid, cost-effective, facile approach applicable to a number of clinical orthopedic scenarios.

Disadvantages

Banked allograft is expensive and, despite the robust septic screens and aggressive intraoperative cleansing cycles, there remains the potential for immune or septic reactions. However, the procedure, as advocated, carries minimal risk of donor-site morbidity from the marrow aspiration. If multiple punctures of the cortex and small volume aspirations are performed, there is also the risk of diluting the yield of osteoprogenitor cells [28]. Current studies are centered on:

- Improvements in the fractionation process to remove contaminating erythrocytes and improve CFU-F numbers;
- Determination of the rate of bone incorporation;
- Whether the living composite confers an advantage in reaching maximum strength in comparison with allograft alone.

This latter issue will ultimately depend on the successful development of a local infiltrative vascular system.

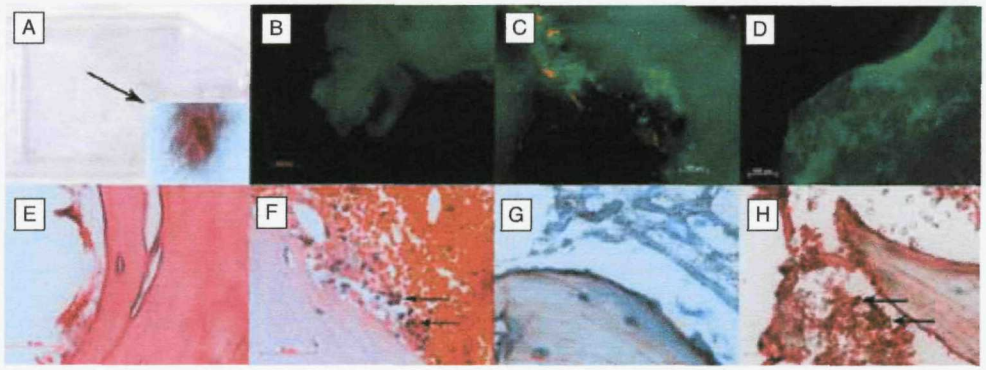
Conclusion

The principles of tissue engineering have been used successfully for the treatment of bone deficiencies in two clinical conditions, whereby human bone marrow stromal cells have been seeded onto washed allograft to form a living composite and impacted into a bony defect. *In vitro* analysis has confirmed cell survival and osteogenic phenotype of the cells.

Future perspective

This facile versatile technique can be tailored to a variety of orthopedic situations and, in particular, offers an exciting potential role in the augmentation of allograft or synthetic graft, used in acetabular and femoral impaction bone grafting. Integral to the attractiveness of this technique is the ability to undertake the whole procedure without tissue leaving the operating room. However, the downside of this is that a highly heterogenous population of cells are seeded onto the graft. Cell-selection machines are used currently in clinical practice and have the ability to remove the red blood cell population from the aspirate, leaving an enriched human bone marrow mononuclear cell fraction. This step could still be performed in theatre, and could increase the proportion of cells that are likely to differentiate along the osteogenic lineage.

Figure 5. Histological and immunohistochemical analysis.



(A) Alkaline phosphatase staining for colony-forming units-fibroblastic (CFU-Fs; inset: ×20 magnification of individual CFU-F). Ethidium homodimer/cell tracker Green staining **(B)** allograft alone (×5). **(C)** Immediate viability of allograft seeded with autologous bone marrow aspirate (×5), and **(D)** at 1 week (×5). Hematoxylin and eosin staining of **(E)** washed allograft alone (×40) and **(F)** seeded allograft (×40). **(H)** Bone sialoprotein immunostaining of seeded allograft (×40) and negative control (×40) **(G)**.

Development of such incremental steps, using tissue-engineering strategies employing appropriate cell populations and scaffolds auger well for further translation of such approaches to create bone formation strategies targeted at the unmet orthopedic needs of many.

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Executive summary

- Reconstitution of extensive bone loss remains a difficult problem for orthopedic surgeons.
- Seeding human bone marrows stromal cells onto highly washed morselized allograft is an innovative technique to aid the reconstitution of substantial bone loss when the volume of graft needed exceed volumes provided by autograft alone.
- The augmentation of graft with mesenchymal populations has been shown to survive the impaction process, converting a loose aggregate into a viable cell composite graft with comparable density to the cancellous bone that it has replaced, without the need for additional metallic support and avoidance of infection.
- All steps of the procedure occur in theatre under sterile conditions and, therefore, avoid the potential increased risks of contamination from removing tissue to a laboratory for cell selection and culture expansion.
- This facile versatile technique can be adapted to a number of different orthopedic scenarios when dealing with the problematic field of reconstituting extensive areas of bone loss.

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